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14. ABSTRACT

A new paradigm for the proliferation and metastasis of breast cancer posits a rare tumor stem cell with low proliferative index and high self-renewing potential. Like its normal counterpart, the tumorigenic stem cell gives rise to transit-amplifying daughters of high clonogenic potential. These in turn lose clonogenic potential as they follow a dysregulated differentiation program into bulk tumor.

The principal hypothesis which this proposal addressed is that the bulk of breast cancer tumor cells arise from rare aberrant stem cells that share functional and phenotypic characteristics with normal tissue stem cells, including high multidrug resistance (MDR) transporter activity. Our studies were based on those of Clarke et al who isolated a rare and highly tumorigenic subset of breast cancer (BrCa) cells on the basis of expression of surface adhesion molecules (CD44+ and CD24low). In this proposal we addressed the hypothesis that these cells (or a subset thereof) have high expression of the MDR transporter ABCG2 and other stem-cell associated markers.

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Introduction

A new paradigm for the proliferation and metastasis of breast cancer posits a rare tumor stem cell with low proliferative index and high self-renewing potential. Like its normal counterpart, the tumorigenic stem cell gives rise to transit-amplifying daughters of high clonogenic potential. These in turn lose clonogenic potential as they follow a dysregulated differentiation program into bulk tumor.

The principal hypothesis which this proposal addressed is that the bulk of breast cancer tumor cells arise from rare aberrant stem cells that share functional and phenotypic characteristics with normal tissue stem cells, including high multidrug resistance (MDR) transporter activity. Our studies were based on those of Clarke et al who isolated a rare and highly tumorigenic subset of breast cancer (BrCa) cells on the basis of expression of surface adhesion molecules (CD44+ and CD24low). In this proposal we addressed the hypothesis that these cells (or a subset thereof) have high expression of the MDR transporter ABCG2 and other stem-cell associated markers.

Body

The objectives of this proposal were to 1) Evaluate CD45-/CD44+/CD24low cells in previously obtained cryopreserved pleural effusions from patients with BrCa; 2) Determine the level of activity of the MDR pump, ABCG2, on CD45-/CD44+/CD24low cells.

Key Research Accomplishments. The key accomplishments of this concept award are illustrated with exemplary data below. Many of the findings have been published in the appended manuscripts or presented at scientific meetings as detailed below.

- Performed seven-color flow cytometry on pleural effusion samples from 30 breast cancer patients. The panel included the stem/progenitor markers CD90, CD117 and CD133, the adhesion molecule CD44, the differentiation markers cytokeratin and CD45, and the MDR transporter ABCG2 (breast cancer resistance protein). An average of 2.5 million events were acquired for each sample (min= 200,000, max= 6,000,000).

- Demonstrated the presence of rare stem and progenitor cell fractions within the CD44+ population as determined by marker expression and physical properties (cell size and morphologic complexity).

iCytokeratin Expression on CD45-CD44/24 Subsets in Malignant Effusion

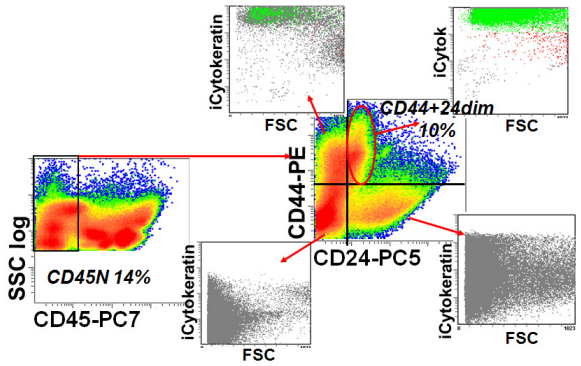


Figure 1. CD44+ 24- tumor cells are cytokeratin dim and have low forward scatter. CD44 and CD24 expression were determined in CD45 negative (nonhematopoietic) cells from a malignant effusion. Intracellular cytokeratin expression and forward light scatter were measured in each subpopulation. The CD44+ CD24- negative subset, found to be enriched in tumorigenic cells by Clarke et al expressed dim cytokeratin and were of small size as indicated by low forward light scatter. We also determined populations positive for the multiple drug resistance transporter ABCG2. ABCG2+ cells are color evented in green and were limited to the CD44+ CD24 negative or dim populations.

CD133/iCytokeratin Expression on CD45-CD44/24 Subsets in Malignant Efffusion

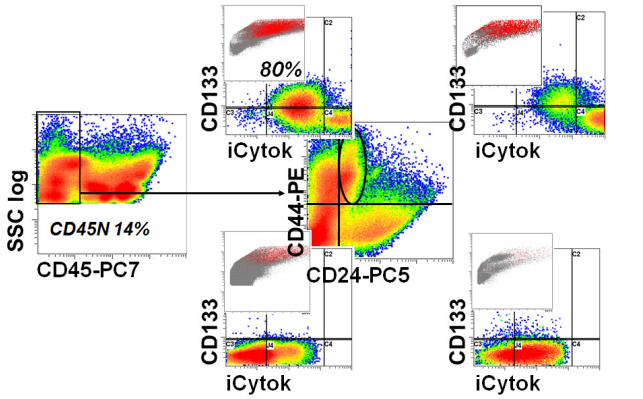


Figure 2. Expression of the progenitor cell marker CD133. CD44 and CD24 expression were determined in CD45 negative (nonhematopoietic) cells from a malignant effusion. Intracellular cytokeratin and CD133 expression measured in each subpopulation. Forward (x-axis) and side (y-axis) light scatter are shown in the inset panels. The CD44+ CD24- and dim populations encompass virtually all of the CD133+ cells.

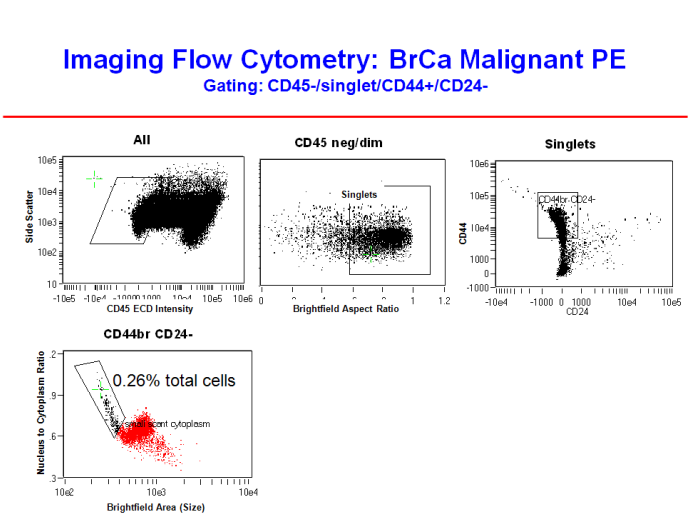


Figure 3. Imaging Flow Cytometry performed on a Malignant Effusion. Cells were stained with antibodies directed against CD45, CD44 and CD24. Imaging flow cytometry was performed using an Amnis ImageStream 100. The CD44+ CD24- population was evaluated for morphological characteristics (cell size, nucleus to cytoplasm ratio). This population was considerably smaller than unselected tumor cells (not shown) and contained a small population (0.26% of total cell) of small cells with very scant cytoplasm. Images of these cells are visualized in Figure 4.

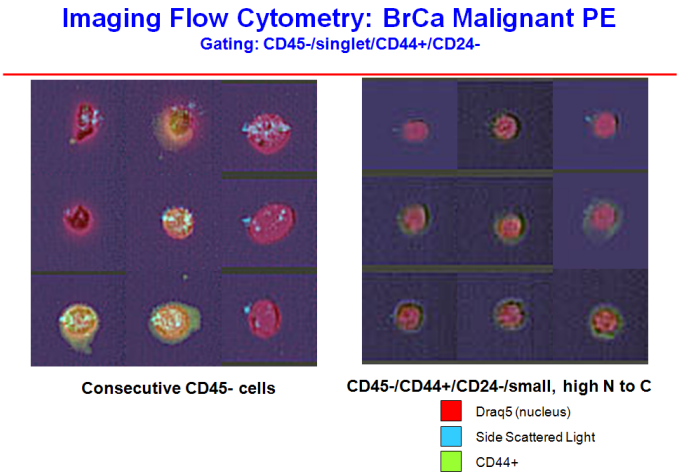
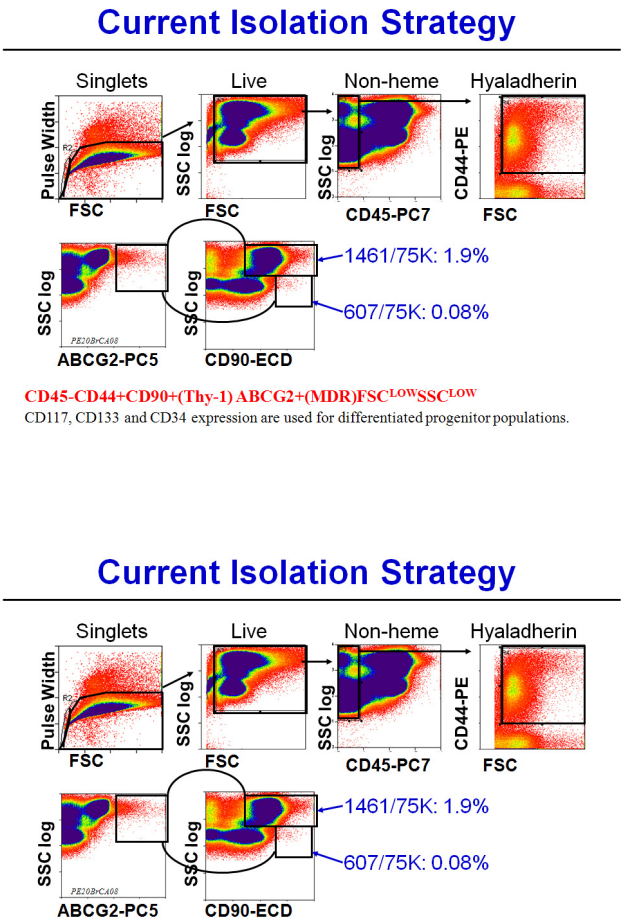


Figure 4. Imaging Flow Cytometry of Unselected CD45- tumor cells, and the high nucleus:cytoplasm population of CD44+ CD24- cells.

- Demonstrated the expression of the MDR transporter ABCG2 on a proportion of stem and progenitor like breast cancer cells.



Current Isolation Strategy
Singlets
Live
Non-heme
Hyaladherin
Pulse Width
SSC log
FSC
CD45-PC7
CD44-PE
1461/75K: 1.9%
607/75K: 0.08%
ABCG2-PC5
CD90-ECD
CD45-CD44+CD90+(Thy-1) ABCG2+(MDR)FSC^LOWSSC^LOW
CD117, CD133 and CD34 expression are used for differentiated progenitor populations.

Figure 5. Isolation strategy for ABCG2+ CD90+ tumor stem and progenitor populations. The top panels shows the strategy in which we gate on singlets (eliminating doublets and cell clusters), exclude apoptotic cells, exclude CD45+ hematopoietic cells, and include only CD44+ cells. Cells expression the ABCG2 transporter are further subsetted into CD90+ high side scatter (putative tumor progenitor cells) and CD90+ low side scatter (putative tumor stem cells). Our subsequent work has demonstrated that both CD90+ cells with and without constitutive MDR transporter expression are tumorigenic. Our current thinking is that the CD44+ CD90+ population, although not uniquely tumorigenic, is uniquely therapy resistant.

- Measured MDR function by Rhodamine 123 and Hoechst 33342 transport in pleural effusion cell subsets. Constitutive transporter activity was confined to a population of cells expressing stem cell markers and having low morphologic complexity.

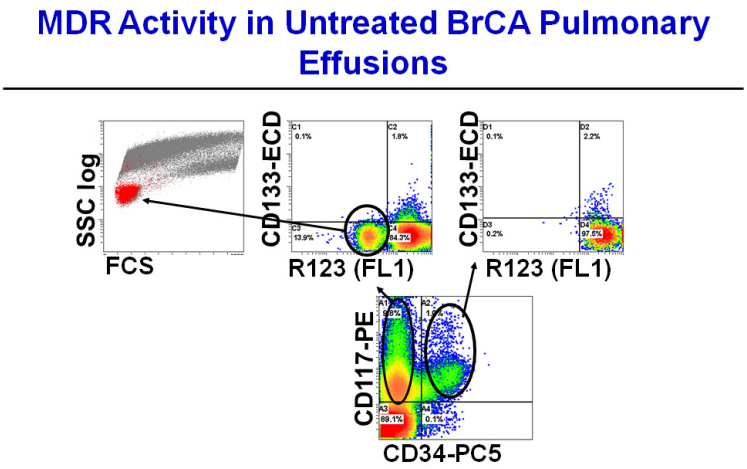


Figure 6. Functional Measurement of MDR Activity by Rhodamine 123 (R123) Efflux. A malignant effusion was gatedfor CD45- cells (as shown in Figure 5) and subsetted by the stem/progenitor markers CD117 and CD34. The CD34+ subset (normal endothelial cells?) remained R123 bright after dye loading and 30 minutes in culture. The CD117+ (stem cell factor receptor+ tumor stem cell) fraction contained a significant fraction of cells with the constitutive ability to efflux the MDR substrate R123. These cells were of uniform low light scatter, indicating a homogeneous population of small cells. CD117 and CD90 are only rarely coexpressed on tumor stem cells. One marker usually predominates over the other in individual patient samples.

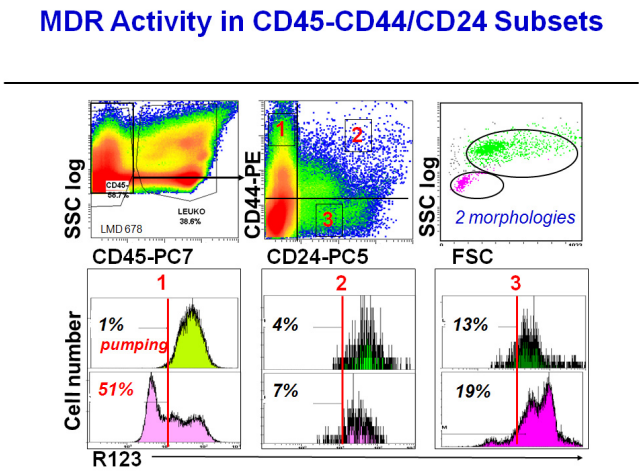


Figure 7. R123 transport in CD45- cells subsetted on the basis of CD44 and CD24. In this malignant effusion sample, cells subsetted by CD44 CD24 expression were further divided into simple (pink) and complex (green) morphologies on the basis of light scatter. R123 efflux was limited to CD44+ CD24- cells of simple morphology.

R123dull/Ho33342 "SP" Have Resting Morphology

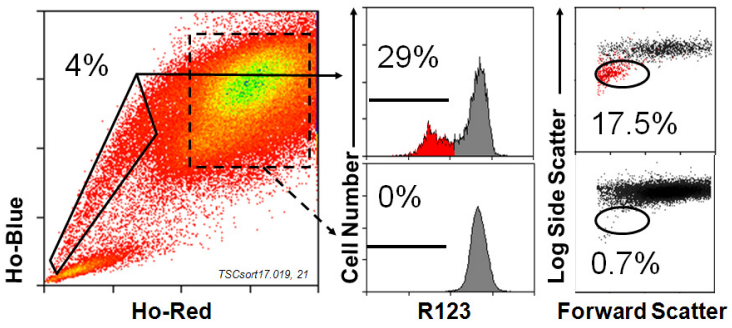


Figure 7. Simultaneous measurement of Hoechst 33342 and R123 Efflux in a primary lung tumor. Although this assay was performed on a freshly isolated lung adenocarcinoma, it demonstrates that R123 transporting cells are s subset of the the Hoechst effluxing "Side Population." Only the "double transporting" cells, color evented red were of simple morphology. SP negative cells do not transport R123 (bottom panels).

Reportable Outcomes

Manuscripts

1. Donnenberg VS, Donnenberg AD. Multiple Drug Resistance in Cancer Revisited: The Cancer Stem Cell Hypothesis. Journal of Clinical Pharmacology. 45:872-877, 2005.

2. Donnenberg VS, Luketich JD, Landreneau RJ, DeLoia JA, Basse P, Donnenberg AD. Tumorigenic Epithelial Stem Cells and Their Normal Counterparts. Ernst Schering Res Foundation Workshop, In Press, 2006.

3. Donnenberg VS, Landreneau RJ, Donnenberg AD. Tumorigenic Stem and Progenitor Cells: Implications for the Therapeutic Index of Anti-cancer Agents. Journal of Controlled Release. In Press, 2007.

Abstracts and Presentations

1. Vera S Donnenberg, Julie A DeLoia, James D Luketich, Albert D Donnenberg. P-Glycoprotein (MDR1) and ABCC1 (MRP) Activity Correlate with R123 Transport in T-Cell Subsets. 16th Annual Meeting, American College of Clinical Pharmacology, October 2004, Phoenix,AZ. Journal of Clinical Pharmacology 2004.

2. Albert D. Donnenberg, Vera S. Donnenberg. Tumor Stem Cells. Clinical Immunology Society Flow Cytometry Course. Long Beach CA, October 2004.

3. Vera S. Donnenberg, Albert D. Donnenberg. Multiple Drug Resistance (MDR) Transporters in Cancer Stem Cells. Great Lakes International Flow Cytometry and Cytology Association, Windsor, OT, Canada. October 2004.

4. Albert D. Donnenberg. Cancer Stem Cells. West Penn Hospital Oncology Seminar Series: Cancer Stem Cells April 8, 2005.

5. Vera S. Donnenberg, Adam M. Brufsky, Neil A. Christie, James D. Luketich, Julie A. DeLoia, Jeffrey A. Romoff, Albert D. Donnenberg. Constitutive Multiple Drug Resistance in Tumor Stem Cells. AACR April 16-20, 2005, Anaheim, Orange County, CA.

6. Albert D. Donnenberg. Cancer Stem Cells: The Root of Cancer. Session Co-Chair. DOD Era of Hope Meeting. Philadelphia PA June 9, 2005.

7. Albert D. Donnenberg, Vera S. Donnenberg. Detection of Putative Breast Cancer Tumor Stem And Progenitor Cells in Primary and Metastatic Lesions. DOD Era of Hope Meeting. Philadelphia PA June 9, 2005.

8. Albert D. Donnenberg. Cancer Stem Cells. Roswell Park Cancer Institute, Buffalo NY, June 26, 2005.

9. VS Donnenberg, JD Luketich, JA DeLoia, NA Christie, RJ Landreneau, AM Brufsky, AD Donnenberg. Constitutive Multiple Drug Resistance In Stem Cells Isolated From Epithelial Tumors Precedes Exposure To Cytotoxic Therapy. 17th Annual Meeting,

American College of Clinical Pharmacology, October 2005, Bethesda, DC. Journal of Clinical Pharmacology 2005.

10. VS Donnenberg, L Niedernhofer, P Basse, RJ Landreneau, JD Luketich, AD Donnenberg. Preexisting Multiple Drug Resistance in Tumorigenic Stem Cells. Keystone Symposium Stem Cells, Senescence and Cancer. Biopolis, Singapore. October 26, 2005.

11. Vera S. Donnenberg and Albert D. Donnenberg, Rodney J. Landreneau, Neil A. Christie, Julie A. DeLoia, Robert Edwards, Laura Niedernhofer, Adam M. Brufsky, J. Peter Rubin, Bruno Peault, Per Basse, Steven Badylak, James D. Luketich. Regenerative Medicine and Cancer: The cancer stem cell paradigm. World Congress on Tissue Engineering and Regenerative Medicine. Pittsburgh, PA. April 25, 2006.

12. Vera S. Donnenberg, Laura Niedernhofer, James L. Luketich, Rodney J. Landreneau, Julie A. DeLoia, Per Basse, Albert D. Donnenberg. Tumorigenic Stem Cells from Therapeutically Naïve Human Epithelial Cancers Express the Drug Resistance Transporter ABCG2. International Society for Stem Cell Research. Toronto, Ontario, CA. June 29, 2006.

13. Albert D. Donnenberg, Vera S. Donnenberg. Cancer Stem Cells and Multiple Drug Resistance. Ernst Schering Symposium on Cancer stem cells: Novel concepts and prospects for tumor therapy. Berlin, Germany, November 2006.

Patents and licenses applied for and/or issued: None
Degrees obtained that are supported by this award: None
Development of cell lines, tissue or serum repositories: None
Informatics such as databases and animal models, etc.: None

Funding received based on work supported by this award:

Grant Title:	Expression of TRPS-1 as a Function of Breast Cancer Tumor Differentiation State
Role in Project	PI
Percent Effort:	10%
Years Inclusive:	1/1/2006-12/31/2007
Direct Costs:	
Source:	Sanofi-Aventis

Grant Title:	CMDRP Era of Hope Scholar Award BC044784 Breast Cancer Stem Cells: A Novel Therapeutic Target
Role in Project	Co-I (V.S. Donnenberg, PI)
Percent Effort:	25%
Years Inclusive:	4/01/05-3/31/10
Direct Costs:	
Source:	DOD

Employment or research opportunities applied for and/or received based on experience/training supported by this award: Vera S. Donnenberg received the CDMRP Era of

Hope Scholar Award based on pilot studies performed under this award. Yvonne Chao, (MD, Ph.D. candidate, University of Pittsburgh Medical Scientist Training Program) and Brian Zitelli (Pharm. D. candidate, University of Pittsburgh School of Pharmaceutical Sciences) have both based their doctoral dissertation work on studies piloted by this award.

Conclusions

The studies piloted in this concept award provided the first evidence that a proportion of breast cancer stem cells express functional multiple drug resistance transporters. They have provided the basis for ongoing studies on the tumorigenicity of isolated breast cancer stem cells, studies of circulating breast cancer stem cells, chemotherapy and radiation resistance in breast cancer stem cells, and breast cancer stem cells as a potential target of immunotherapy.

Appendix

The following Manuscripts are Appended:

Donnenberg VS, Donnenberg AD. Multiple Drug Resistance in Cancer Revisited: The Cancer Stem Cell Hypothesis. Journal of Clinical Pharmacology. 45:872-877, 2005.

Donnenberg VS, Luketich JD, Landreneau RJ, DeLoia JA, Basse P, Donnenberg AD. Tumorigenic Epithelial Stem Cells and Their Normal Counterparts. Ernst Schering Res Foundation Workshop, In Press, 2006.

Tumorigenic Epithelial Stem Cells and Their Normal Counterparts

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Abstract
ABC transporters are highly conserved and represent a major protective mechanism for barrier tissues as well as adult tissue stem cells. Emerging data support the existence of a cancer stem cell that shares features of tissue stem cells, including the ability to self-renew and undergo dysregulated differentiation. Here we show that a rare population of cells coexpressing MDR transporters and stem cell markers is a common feature across therapy naïve epithelial cancers as well as normal epithelial tissue. Although MDR+ and negative candidate tumor stem and progenitor populations were all capable of generating highly anaplastic transplantable human tumors in NOD/SCID mice, only resting stem-like breast cancer cells were capable of incorporating into murine ductal tissue with high efficiency. The finding that rare cells bearing stem cell markers and having intrinsic MDR expression and activity are already present within the tumorigenic compartment before treatment with cytotoxic agents is of critical importance to cancer therapy. Just as damaged normal epithelial tissues regenerate after chemotherapy by virtue of highly protected resting tissue stem cells, the existence of malignant counterparts in therapy naïve epithelial cancers suggests a common mechanism by which normal and tumor stem cells protect themselves against toxic injury.

Background
Multiple drug resistance (MDR) was early recognized as a barrier to cancer therapy (Biedler et al., 1970). The common mechanism responsible for cross-resistance to multiple structurally unrelated agents was determined to be reduced cellular permeability (Ling and Thompson, 1974), mediated by a family of highly conserved proteins known as

ATP binding cassette (ABC) transporters (Leslie et al., 2005). Although ABC transporter expression is recognized as a significant cause of chemotherapy resistance, the prevalent paradigm understands MDR in cancer to result from drug-mediated selection of cells with ABC transporter gene amplification (Chen et al., 2002) or regional gene activation (Wang et al., 2006). More recently it has become apparent that normal adult tissue stem cells, including hematopoietic (Udomsakdi et al., 1991, Chaudhary and Roninson, 1991, Goodell et al., 1996), airway (Giangreco et al., 2004), pituitary (Chen et al., 2005), small intestine (He et al., 2005) and testes (Riou et al., 2005) express high levels of MDR transporter activity. Persistence of tissue stem cells is essential to tissue maintenance and repair, and constitutive MDR activity is though to be one of several mechanisms by which normal tissue stem cells protect themselves form from toxic insults, including those resulting from damage by chemotherapeutic agents (Donnenberg and Donnenberg, 2005). A dramatic example can be found in chemotherapy-induced alopecia, which results from damage to the rapidly cycling progenitor cells of the hair follicle (Paus and Cotsarelis, 1999, Alonso and Fuchs, 2003). However, alopecia is reversed on cessation of therapy because the common precursor of the four distinct cell types within the follicle, as well as skin epithelial cells themselves, are all derived from a resting epithelial stem cell (Rendl et al., 2005), which is protected by constitutive MDR activity (Yano et al., 2005).

The cancer stem cell paradigm (Fiala 1968, Hamburger and Salmon, 1977, Reya et al. 2001, Dick 2003, Al-Hajj et al. 2004, Donnenberg and Donnenberg, 2005, Dick and Lapidot, 2005, Wicha et al., 2006, Polyak and Hahn, 2006) envisions the cancer-initiating cell as a genetically damaged tissue stem cell, or a more mature cell which has reacquired stem cell attributes through mutation. The unique insight which we derive from the study of adult tissue stem cells is that drug resistance is a normal self-protective mechanism which may be retained by the nascent neoplasm upon transformation of the tissue stem cell. The notion that the cancer stem cell, or a subset of these cells, may have constitutive drug resistance agrees with the observation that cancers often recur after apparently successful therapy.

Results

ABGG2+ cells are present in therapy naïve tumor and normal lung and express stem/progenitor markers. Stem cells from a variety of epithelial tissues have been enriched by sorting for cells with constitutive MDR transporter activity. To investigate expression of the MDR transporter ABCG2 in freshly isolated therapy-naïve epithelial tumor cells, single cell suspensions were prepared from solid tumors, malignant ascites and effusions. Normal lung tissue was also investigated as a positive control. A population of non-hematopoietic, cytokeratin+, ABCG2+ cells was present at low frequency in both neoplastic and normal tissues (Figure 1).

Figure

PLACE FIGURE 1 HERE

All newly diagnosed untreated epithelial tumors contained a rare subset of CD45- cytokeratin^{dim} ABCG2+ cells (0.43 ± 0.57 of CD45- cells, Mean ± Standard Deviation). ABCG2+ cytokeratin^{dim} cells also expressed CD44 (69 ± 18%), and the stem/progenitor markers CD90 (62 ± 20%), CD117 (34 ± 23%) and CD133 (25 ± 23%). Eight ± 5 % of ABCG2+ cells (0.03% of CD45- cytokeratin^{dim}) had low forward and side light scatter profiles compatible with small resting morphology.
None of these markers, alone or in combination, were able to distinguish normal lung from primary epithelial tumors. In contrast ABCG2+ cells from previously untreated metastatic cancers (effusions and ascites) had significantly lower proportions of CD90+ and CD117+ cells (p=0.034, and 0.011, respectively), and a higher proportion of CD133+ cells (p=0.015) than either normal lung or primary tumor. These data demonstrate that therapy-naïve epithelial tumors contain a rare subpopulation MDR positive, stem cell marker positive cells, a phenotype shared with an equally rare subset present in normal lung tissue. Further, this subpopulation was detectable in malignant effusions and ascites, sites unlikely to harbor normal tissue stem cells. Together these data suggest that stem cells within the tumor are not simply normal stem cells engaged in wound healing, and that these tumor cells share mechanisms with normal tissue stem cells that may equally confer resistance to cytotoxic therapy.

ABC transporters are constitutively active in a small subset of therapy naïve tumor cells. Functional measurement of ABC transporter activity is important, since expression and activity are not always well correlated (Webb et al., 1996). In Figure 2 we show simultaneous transport of the MDR transporter substrates Hoechst 33342 and R123, in freshly isolated cells from a therapy naïve non-small cell lung tumor. The SP phenotype (ABCG2- and ABCB1-mediated transport) comprised 4% of non-hematopoietic cells, 29% of which had concomitant R123 efflux (ABCB1 transport, Figure 2, color-evented red). None of the SP negative cells (Hoechst bright) transported rhodamine.

Figure

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Virtually all of the *dual transporting* cells exhibited low light scatter, consistent with a resting morphology. The ABC transporter specificity of dye efflux was demonstrated with the ABCG2 specific inhibitor fumitremorgin, which abrogated 75% of the SP phenotype. CD90+ cells were present in both SP+ and SP negative fractions, indicating that not all cells bearing this stem cell marker have MDR activity. Further, when we examined R123 efflux among the CD45- CD117+ subset of untreated ovarian and lung tumor cells, MDR activity was restricted to the subset with low morphologic complexity and G1/G0 cell cycle phase (n=5, data not shown). Taken together, these data demonstrate that resting stem cell marker positive tumor cells with low morphologic complexity express both ABCG2 (breast cancer resistance protein 1) and ABCB1 (P-glycoprotein) and exhibit the highest constitutive MDR activity.

Stem and progenitor cell candidate populations are tumorigenic. In normal tissue, self-renewal, hence the capacity for theoretically unlimited growth, resides in the stem

cell population. Progenitor cells, the progeny of stem cells, have high proliferative capacity but limited ability to self-renew (Watt 1998). Two populations of CD45- cells, representing candidate stem and progenitor fractions, were sorted from an untreated freshly isolated non small cell lung tumor and tested for tumorigenicity in NOD/SCID mice. Three groups of 3 mice each were injected i.v. with 15,000 cells: 1) Stem cell-like CD45- CD90+ CD133- HEA-; 2) Progenitor cell-like CD45- CD90- CD133+ HEA+; or 3) Unsorted tumor. Mice were sacrificed between days 132 and 294, when they exhibited changes in behavior or appearance. All mice injected with candidate stem or progenitor fractions developed lung tumors, many of which were widely metastatic to other organs (Table 1).

Table

PLACE TABLE 1 HERE

Two of three mice injected with unsorted tumor cells also developed lung tumors, one of which was grossly apparent and one of which was microscopic. Figure 3 shows imaging flow cytometry performed on this sample concurrent with cell sorting. The CD45- CD90+ stem fraction shown in panel A comprised 5% of CD45- singlet cells and were of uniform small morphology with high nucleus to cytoplasm ratio. In contrast, CD90- cells comprised the vast majority of CD45- tumor cells and were heterogeneous with respect to morphology. These data demonstrate that both small morphologically bland cells bearing the stem cell marker CD90 in the absence of the epithelial differentiation marker HEA (stem cell candidate), and size heterogeneous CD90- CD133+ HEA+ cells (progenitor candidate) are tumorigenic.

Figure

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Transporter positive and negative cells are tumorigenic. In order to determine tumorigenicity of ABCG2 protected fractions, particularly the resting fraction, ABCG2 positive and negative CD90+ cell populations were sorted from a recurrent breast cancer pleural effusion. These populations were defined within the CD45 negative CD44+ fraction shown previously to contain tumorigenic breast cancer cells (Al-Hajj, 2003). The tumorigenicity of low light scatter (resting) and high light scatter (differentiated) tumor populations were examined separately. For each of the four fractions, the total cells recovered after sorting were divided into 4 equal aliquots and injected into the mammary fat pad admixed with 10,000 heavily irradiated (10,000 rads) sorted CD45- tumor cells suspended in Matrigel. Thus, animals received 43-58 sorted cells from the resting cell fractions, and 633-13,200 cells from the more prevalent high scatter fractions (Figure 4). Additionally, two groups of two mice were injected at four sites each with 10,000 sorted CD45- unirradiated or irradiated tumor cells, respectively. All sorted CD90+ cell fractions generated tumors, even the rare resting fractions where 43 and 58

cells were injected. The proportion of mice developing tumors and the day of sacrifice are shown in Figure 4, with characteristic immunohistochemical staining for human cytokeratins. Tumors grew slowly and were first palpable at 5-10 months. Irradiated cells were not tumorigenic. None of the mice injected with sorted CD45- cells evidenced tumors at the time that mice injected with CD90+ cells were sacrificed. However, small tumors were observed in 2 of 8 sites injected with CD45- cells when the experiment was terminated at day 371. Tumors were poorly differentiated with atypical nuclei.

Figure

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Despite the homogeneity of the injected human cell populations, flow cytometry of tumor xenografts revealed a heterogeneity strikingly similar to the clinical isolate, regardless of the sorted population of origin. Figure 5 shows a detailed flow cytometric analysis of the freshly isolated pleural effusion, sorted population #4 (CD45- CD44+ CD90+ ABGG2-), and a tumor that was harvested 204 days after injection of this population. With the exception of human CD45+ lymphohematopoietic cells, which were prevalent in the effusion and absent in the xenograft, all major effusion populations were observed in the xenograft. Interestingly, the injected population was present at a similar frequency in the original tumor and the effusion. This provides evidence for self-renewal and expansion of the CD90+ ABCG2- fraction, since 13,200 cells were injected and the resulting tumor measured 3mm. Importantly, the injected ABCG2 negative cells gave rise to ABCG2+ cells which were seen in both CD90+ and negative fractions, as they were in the fresh clinical isolate.

Figure

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To determine whether tumor xenografts could be passaged, tumors #1 and 4, harvested on day 302, were disaggregated and sorted into CD45- CD44+ HEA+ CD90+ ABCG2- and CD45- CD44+ HEA+ CD90+ ABCG2+ fractions. Sorted cells were admixed with 10,000 irradiated unsorted tumor cells, suspended in Matrigel and injected into the mammary fat pads of NOD/SCID mice (2 animals/fraction). The CD90+ ABCG2- fraction from tumors 1 and 4 yielded 875 and 1285 cells per injection site, respectively. The CD90+ ABCG2+ fraction from tumors 1 and 4 yielded 43,750 and 30,000 cells per injection site, respectively. Mice were sacrificed on days 129 and 231 with 3-8 mm subcutaneous tumors at the injection sites of all fractions.

Discussion

In this report we have demonstrated the existence of a rare population of CD44+ cytokeratin+ ABCG2+ CD90+ cells across a spectrum of previously untreated epithelial cancers, as well as in normal lung tissue. A proportion of these cells has resting

morphology and coexpresses the stem/progenitor markers CD117 and CD133. The unexpected finding that the ABCG2+ population and its subsets are detected at similar frequency in normal and neoplastic tissues, as well as across epithelial cancers from different organs, suggests that elements of the normal epithelial stem cell function and differentiation are universally retained after neoplastic transformation. In contrast, great variability was seen in expression of maturation/differentiation markers (cytokeratin, MUC-1, HEA) between tumors from different organs, reflecting the different tissues of origin (data not shown). Interestingly, the frequency of CD90+ and CD117+ cells (candidate stem fraction) was lower, and the frequency of CD133+ cells (candidate progenitor fraction) was higher in untreated metastatic sites (Figure 1).

Sorted CD44+ CD24- breast cancer cells (Al-Hajj, 2003), as well as sorted CD133+ cells from brain tumors (Singh et al., 2004) and prostate cancer (Collins et al., 2005) have previously been shown to be tumorigenic in NOD/SCID mice. Although these studies have been widely quoted as supporting the cancer stem cell hypothesis, they did not attempt to distinguish between stem and progenitor compartments and did not determine whether the tumorigenic fraction was protected by mechanisms common to normal tissue stem cells. In this report we used the markers CD90 and CD133 to identify the stem/progenitor fraction within the CD45- CD44+ compartment. Within this population, low morphologic complexity and the differentiation marker HEA (Table 1) were used to provisionally distinguish between resting stem cells and more differentiated progenitor cells. We found that both stem and progenitor populations are tumorigenic, and both have a subset which expresses the ABC transporter ABCG2. However, only the resting stem cell fraction had a subpopulation with constitutive activity of both ABCG2 and ABCB1 transporters (Figure 2). Further, the stem cell fraction was tumorigenic at very high frequency.

Biologically, the salient finding is that untreated epithelial tumors retain a vestige of the ordered growth and differentiation of the parent tissue, including the persistence of resting stem-like cells (some of which are protected by MDR transporters), a more differentiated tumorigenic progenitor fraction, and their post-mitotic non-clonogenic progeny. Despite the phenotypic heterogeneity of tumorigenic cells, the most critical population from a therapeutic standpoint is the resting stem cell-like population. We hypothesize this population to be as resistant to cytotoxic therapy as its normal counterpart, by virtue of constitutive MDR activity and possibly other protective mechanisms afforded by the niche in which it persists (Arai et al, 2005). This population provides an attractive candidate for the cancer stem cell postulated by Weissman (Reya et al., 2001; Al-Hajj et al., 2004) Dick (Dick, 2003, Dick and Lapidot, 2005) and others: a resting, drug resistant tumor cell which can lay dormant after initially successful therapy, providing a seed for later recurrence and metastasis.

The finding of intrinsic MDR activity within a rare resting tumorigenic population is not explained by the conventional MDR paradigm, which views ABC transporter mediated drug resistance as a trait that tumor cells acquire upon drug exposure through either substrate-driven induction, gene amplification or regional gene activation. By concentrating on freshly isolated therapy naïve clinical isolates, we have demonstrated

that ABC transporter expression and activity is present prior to exposure to cytotoxic agents. Given the central role of MDR transporters in protecting normal tissue stem cells, our data support a broadened interpretation of the cancer stem cell paradigm, and provide a unified explanation for the successes and failures of cytotoxic antineoplastic therapy. Namely, the ultimate target, the MDR protected resting cancer stem cell, is spared along with its normal tissue stem cell counterparts. Since cytotoxic regimens must be designed to minimize irreversible toxicity to normal tissue, the therapeutic index has traditionally been thought of as the differential sensitivity of measurable tumor versus that of the highly protected adult tissue stem cell compartment, which is required for regeneration. Our findings recast this concept as the differential sensitivity of MDR-protected tumor stem cells and their normal tissue counterparts.

Methods

Patient samples. Thirty-four patient samples (tumor, adjacent normal tissue, ascites, and pleural effusions) were acquired under protocols approved by the University of Pittsburgh Internal Review Board. With the exception of the sample described in Figures 4 and 5, all were obtained from patients at the time of tumor resection and prior to cytotoxic or radiation therapy.

Tissue Digestion. Solid tissues were minced with paired scalpels, digested with type I collagenase (4% in RPMI 1640 medium, Sigma Chemicals, St. Louis MO) (Elder and Whiteside, 1992) and disaggregated through 100 mesh stainless steel screens. Ten to 500 million viable cells were recovered from a 5-10 mm3 specimens of tumor or normal lung parenchyma. Pleural effusions and ascites were concentrated, collagenase digested and separated on a ficoll/hypaque gradient.

Staining and Flow Cytometry. Single cell suspensions were stained according to a protocol described in detail elsewhere (Donnenberg and Donnenberg, 2003). Five minutes prior to staining with fluorochrome-conjugated monoclonal antibodies, neat mouse serum (5 uL) was added to each cell pellet to minimize non-specific antibody binding. Prior to cytokeratin staining, cells were stained for surface markers and permeabilized with 0.1% saponin (Beckman Coulter, Fullerton, CA) in phosphate buffered saline with 0.5% human serum albumin. Antibodies and dyes used in these studies included: MUC-1-FITC (BD Biosciences, San Jose, CA, Cat No. 559774), HEA-FITC (Miltenyi Biotech, Bergisch Gladbach, Germany, Cat. No. 12000420), Pan cytokeratin-FITC (Beckman Coulter, Cat. No. IM2356), CD44-PE (Serotec, Oxford, UK, Cat. No. MCA 89PE); CD90-biotin (BD, Cat. No. 555594), Streptavidin-ECD (Beckman Coulter, Cat. No. IM3326), ABCG2-PC5 (Chemicon, Temecula CA, Cat. No. MAB4155PC), CD117-PC7 (Beckman Coulter, Cat. No. IM3698), CD133-APC (Miltenyi Biotech, Cat. No. 120001241), CD45-APCC7 (BD, Cat. No. 557833), Propidium iodide (Calbiochem, La Jolla, CA, Cat. No. 537059), Rhodamine 123 (Sigma Chemicals, St. Louis MO, Cat. No. R8004), Hoechst 33342 (Invitrogen, Carlsbad, CA, Cat. No. H3570), Draq5 (Alexis Biochemicals, Lausen, Switzerland, Cat. No. BOS-889-001-R200). Fumitremorgin was purchased from Alexis, (Cat. No. ALX-350-127). Seven-color analysis was performed using the 3-laser, 9-color CyAn LX cytometer (DakoCytomation, Fort Collins, CO). Sorting and analysis requiring an ultraviolet laser

was performed on a 3-laser 8-color DakoCytomation MoFlo. An effort was made to acquire a total of 5 million cells per sample at rates not exceeding 10,000 events/second. The cytometers were calibrated prior to each use using SpectrAlign beads (DakoCytomation, Cat. No. KO111) and 8-peak Rainbow Calibration Particles (Spherotech, Libertyville, IL, Cat. No. RCP-30-5A). Color compensation matrices were calculated for each staining combination within each experiment using single-stained mouse IgG capture beads (Becton Dickinson, Cat. No. 552843) for each antibody, and single-stained cells for rhodamine 123. Offline analysis was performed using Summit software (DakoCytomation). In all analyses, doublets and clusters were eliminated using forward scatter peak width versus height as a discriminator. Propidium iodide staining was used to eliminate nonviable cells.

Tumor Xenografts. Female NOD.CB17-Prkdcscid/J mice 6-8 weeks of age were purchased from The Jackson Laboratory (Bar Harbor, ME), and housed 5 to a cage in a specific pathogen free environment. Prior to injection of tumor cells, mice were anesthetized by methoxyflurane inhalation. For experiments involving intravenous injection, tumor cells were suspended in 50 uL DMEM, 15% fetal bovine serum and injected into the tail vein. For subcutaneous injection, cells were admixed with sorted CD45- tumor (irradiated with 10,000 rads from a 137Ce source) and suspended in 25 uL ice cold DMEM, 15% FBS plus 25 uL Matrigel (Becton Dickinson). 50 uL of ice cold cell suspension were injected subcutaneously into the mammary fatpads (4 injections/animal). Animals were examined twice weekly for behavioral changes and evidence of tumor.

Statistical Analysis. The frequencies of cells expressing stem cell markers were compared using Student's t-test for 2 groups (2-tailed test). Statistical tests, descriptive statistics and graphic analysis (other than cytometry) were performed using Systat, version 11 (Systat Software Inc, Richmond CA).

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FIGURES

Figure 1. Expression of ABCG2 and stem cell markers on freshly isolated normal lung tissue and therapy naïve malignant cells. To investigate expression of the MDR transporter ABCG2 in freshly isolated therapy-naïve tumor cells, single cell suspensions were prepared from solid tumors (Lung Cancer 7, Ovarian Cancer 3) and malignant ascites and effusions (Lung Cancer 2, Ovarian Cancer 6, Gastric Cancer 1) by mechanical dissection and collagenase digestion. Samples were stained by 7-color flow cytometry for expression of ABCG2, CD45, intracellular cytokeratin, CD44, CD90, CD117 and CD133. An average of 2.5 million events were acquired for each sample (min= 200,000, max= 6,000,000).

The first row shows that gating strategy used in this and subsequent analyses. Forward scatter pulse height (x-axis) and pulse width (y-axis) are used to define singlet cells and eliminate cell clusters. Forward and side light scatter are then used to eliminate debris and dead cells. CD45 expression and side light scatter are used to eliminate hematopoietic cells. The second row shows ABCG2 and cytokeratin expression in the gated population of representative normal lung, lung tumor, metastatic lung pleural effusion and ovarian ascites. The percent of ABCG2+ cytokeratin+ cells are shown. The average frequency of these cells at each site is shown in the first bar graph (error bars = SEM). The remaining bar graphs show the frequencies of cells with resting morphology (lymphoid light scatter), CD44, CD90, CD117 and CD133 expression in the CD45-, ABCG2+, cytokeratin+ population.

Figure 2. ABCG2 and ABCB1 activity in freshly isolated therapy naïve non-small cell lung cancer. Antibody-stained suspended tumor cells were incubated simultaneously with the ABCG2/ABCB1 substrate Hoechst 33342 (8 uM) plus the ABCB1 substrate rhodamine 123 (R123, 0.13 uM) for 90 minutes at 37°C (Bertoncello and Williams, 2004). Hoechst emission was separated using a 510 nm dichroic long pass filter. Blue and red fluorescence were measured with 450-65 nm, and 670-20 nm bandpass filters, respectively. Propidium iodide (PI, 10 ug/mL) was added immediately before sample acquisition. All events were gated on PI excluding (live), non-hematopoietic singlets. Five million events were collected. The leftmost panel shows a small population (4%) of Hoechst 33342-excluding cells in the typical pattern of the Side Population (SP). SP (top panels) and non-SP cells (bottom panels) were further characterized: A proportion of SP cells also excluded the ABCB1 substrate dye R123. These accounted for 29% of the SP cells (color-evented red in the dot plots) and accounted for almost all of the cells with low forward and side light scatter, consistent with a resting morphology (Figure 3). Non-SP cells did not transport R123 and were exclusively of high light scatter. A significant proportion of both SP and non-SP cells expressed CD90, often in combination with epithelial specific antigen, HEA. Coincubation of tumor cells with Hoechst 33342, R123, and the ABCG2 specific inhibitor fumitremorgin (10 uM) resulted in 75% inhibition of the SP phenotype.

Figure 3. CD45- CD90+ cells isolated from primary tumors have small resting morphology. A freshly resected untreated NSC lung Ca was collagenase digested and stained with CD45, CD90 and the nuclear stain Draq5 (5 uM). Virtual sorting was

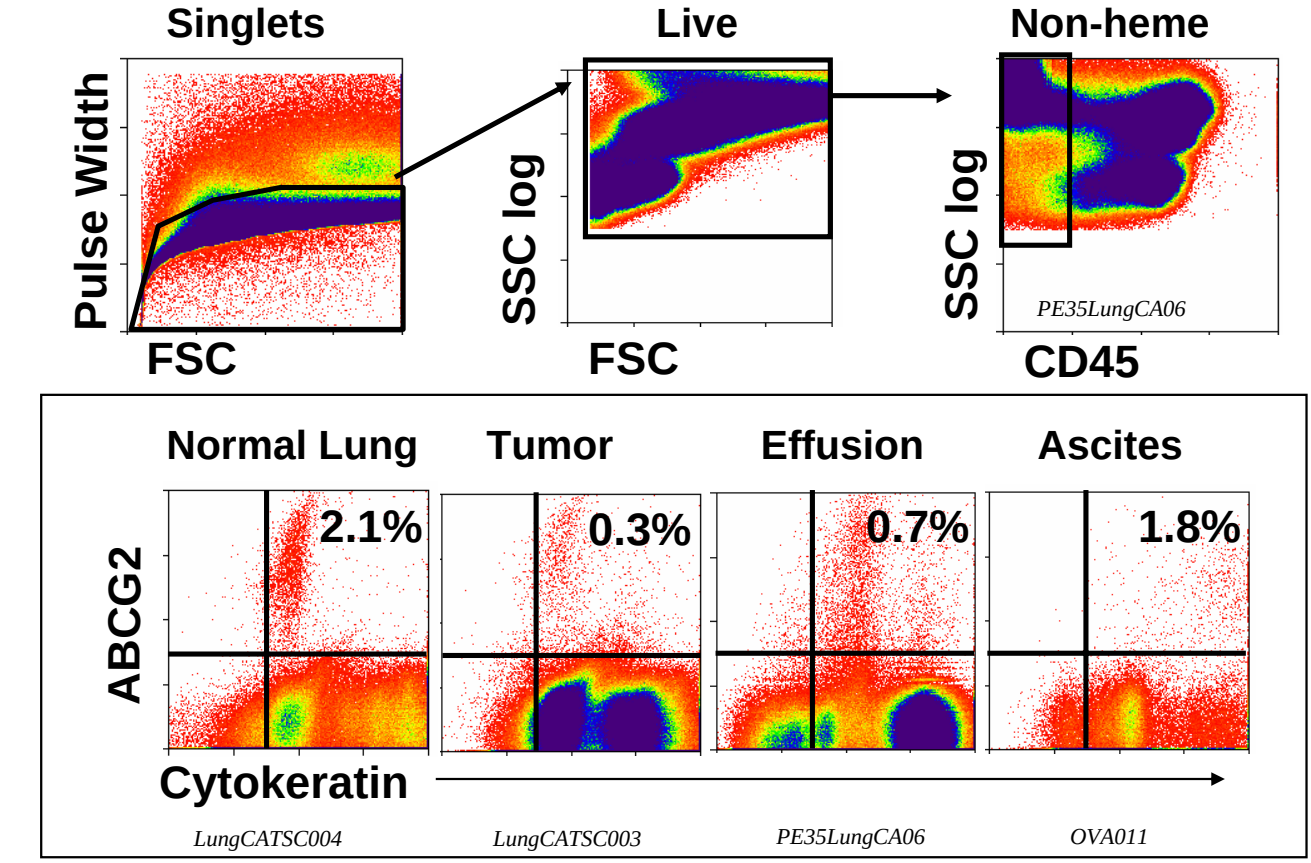
performed using an Amnis ImageStream100 imaging flow cytometer (Amnis Corporation, Seattle WA). All analyzed cells were singlets, as determined by a histogram of brightfield area versus brightfield aspect ratio. Images are composites of brightfield, CD90 (false colored green), and Draq5 (false-colored red). The top panel (A) shows images of nonhematopoietic (CD45 negative) CD90+ cells. The bottom panel (B) shows images from consecutive CD45- CD90- cells. CD90+ tumor cells were small with a relatively high nucleus to cytoplasm ratio.

Figure 4. In vivo tumorigenicity of ABCG2+ and ABCG2- breast cancer cells. Twenty NOD/SCID mice were injected with FACS sorted freshly isolated breast cancer pleural effusion cells as indicated. Sorted cells were admixed with 10,000 heavily irradiated CD45- tumor cells to minimize loss of small numbers of sorted tumor cells. The proportion of mice developing tumors and the days of sacrifice are indicated. Photomicrographs (40X objective, hematoxylin and eosin stain) illustrate histology on tumor xenografts harvested 302 days after injection of sorted cells. Tumors from all fractions were poorly differentiated, with abundant human cytokeratin+ cells in most (not shown), but not all tumors.

Figure 5. Self-renewal and differentiation in a breast cancer tumor stem cell xenograft. The first column shows 7-color flow cytometry performed on the freshly isolated breast cancer pleural effusion which was sorted and injected into NOD/SCID mice. Superimposed are gates identical to those used to sort population 4 (high light scatter, CD45-, CD44+, CD90+, ABCG2-, 13,200 cells injected/mouse). The second column shows the tumor xenograft, which has differentiated substantially, showing light scatter heterogeneity, and the emergence of CD44 and CD90 negative populations. Most importantly, a population of ABCG2+ cells (dashed box, 0.9%) was observed, indicating that MDR expression can be induced in the progeny of ABCG2- cells. Self-renewal can also be seen in the solid boxes (column 2) which indicate the xenograft tumor population falling within the original sort logic used to isolate population 4. Similar to the original pleural effusion, these cells comprised 13.1% percent of the xenograft tumor. However, since they arose from only 13,200 injected cells, the original CD45- CD44+ CD90+ ABCG2- cells expanded substantially within the xenograft tumor. Note: All histograms were gated on singlets (not shown). A total of 6 million effusion cells, and 1.1 million xenograft tumor cells were analyzed.

Table 1. Tumorigenicity of stem and progenitor populations isolated from untreated NSC lung cancer cells. Freshly isolated sorted primary tumor cells (15,000) were injected i.v. into the tail veins of NOD/SCID mice. The experiment was terminated on day 357.

Population	Frequency	Sacrifice Day	Tumor Sites
Stem CD45- CD44+ CD90+ CD133- HEA-	4.36%	272 272 294	Lung, Liver, Spleen Lung Lung, Liver, Spleen, Kidney, Lymph Nodes
Progenitor CD45- CD44+ CD90- CD133+ HEA+	2.93%	205 246 294	Lung Lung, Liver Lung, Liver, Kidney, Lymph Nodes
Unsorted	100%	132 294 357	Lung (microscopic) Lung Negative


Singlets
Live
Non-heme
Pulse Width
FSC
SSC log
FSC
SSC log
CD45
PE35LungCA06
Normal Lung
Tumor
Effusion
Ascites
2.1%
0.3%
0.7%
1.8%
ABCG2
Cytokeratin
LungCATSC004
LungCATSC003
PE35LungCA06
OVA011

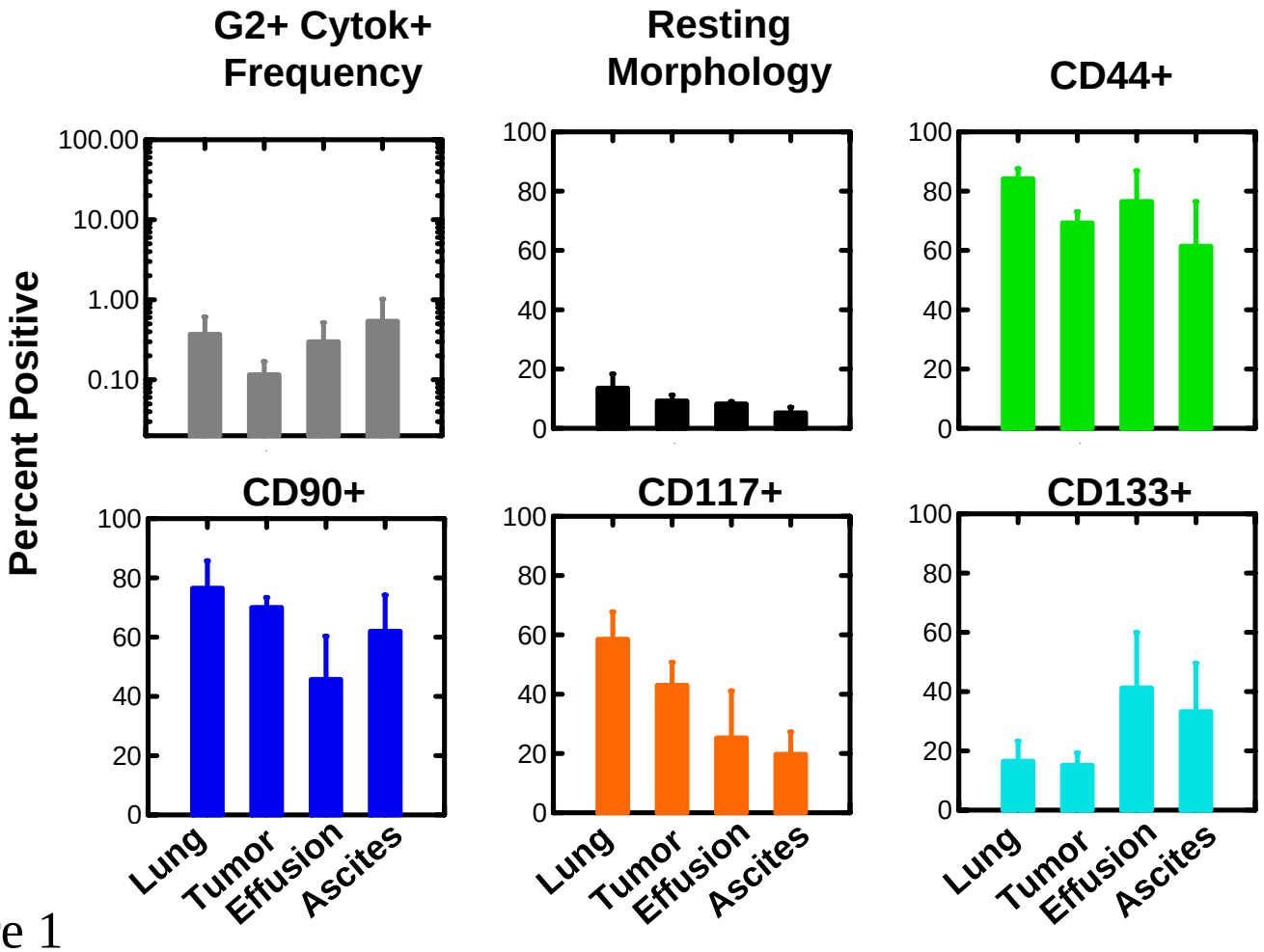

G2+ Cytok+ Frequency
Resting Morphology
CD44+
Percent Positive
100.00
10.00
1.00
0.10
100
80
60
40
20
0
CD90+
CD117+
CD133+
Lung
Tumor
Effusion
Ascites

Figure 1

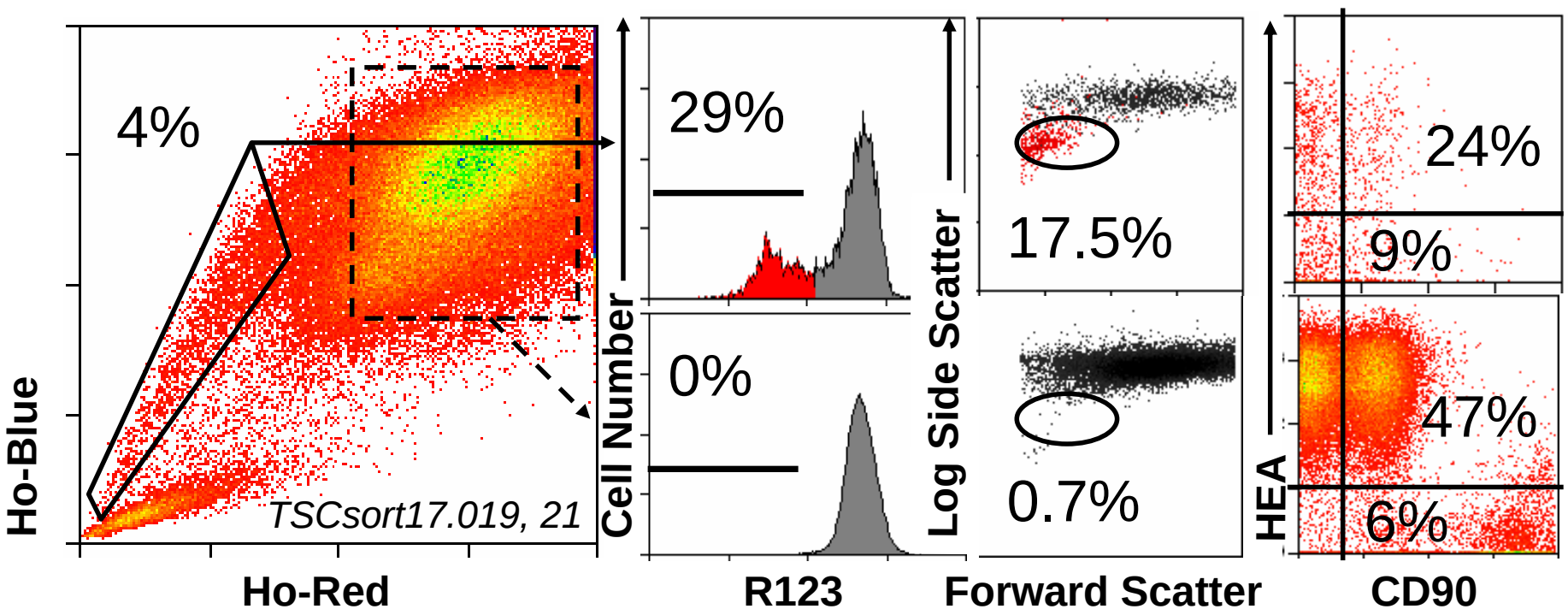

4%
TSCsort17.019, 21
Ho-Blue
Ho-Red
29%
0%
Cell Number
R123
17.5%
0.7%
Log Side Scatter
Forward Scatter
24%
9%
47%
6%
HEA
CD90

FIGURE 2

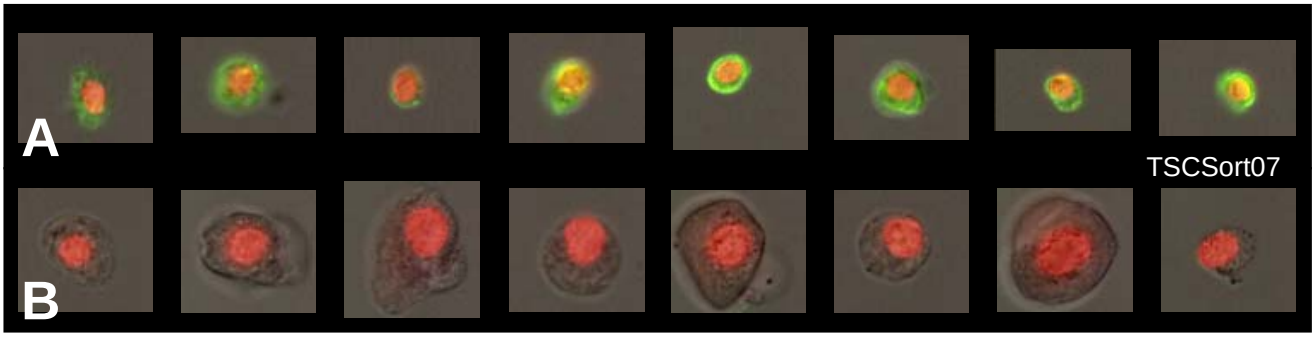
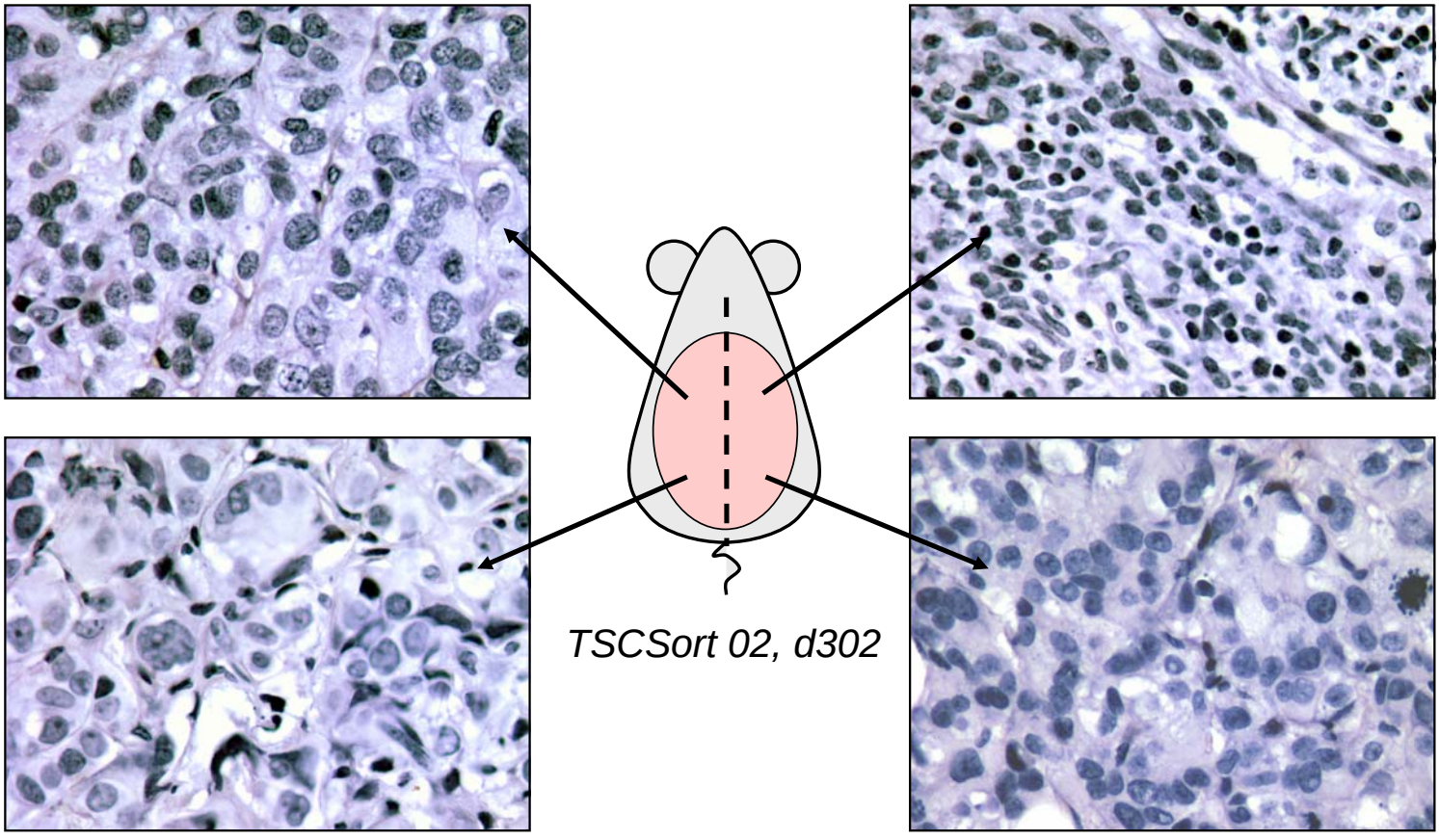


Figure 3

Population #1
CD90±G2+ resting
58 cells/injection
3/4 sites (d160-302)

Population #2
CD90+G2- resting
43 cells/injection
4/4 sites (d160-302)



TSCSort 02, d302

Population #3
CD90±G2+ large
633 cells/injection
3/4 sites (d160-302)

Population #4
CD90+G2- large
13,200 cells/injection
4/4 sites (d160-302)

10,000 Irradiated CD45-: 0/8 sites

10,000 CD45-: 2/8 sites (d371)

Figure 4

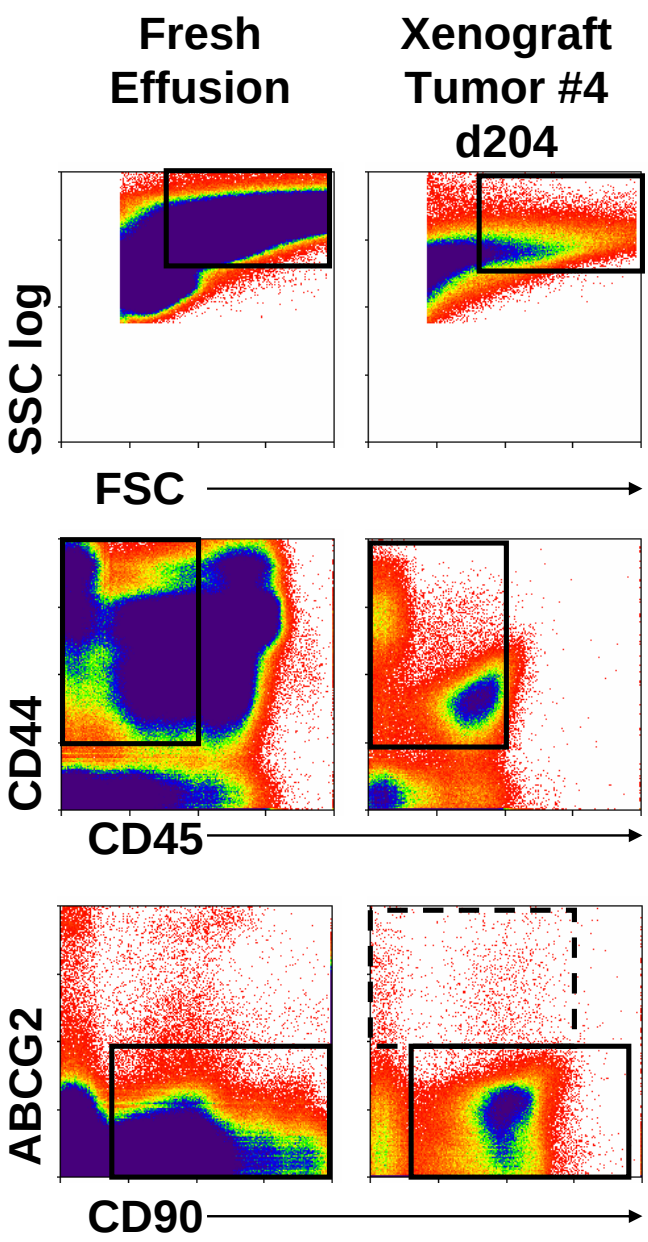

Fresh Effusion
Xenograft Tumor #4 d204
SSC log
FSC
CD44
CD45
ABCG2
CD90

Figure 5

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Tumorigenic stem and progenitor cells: Implications for the therapeutic index of anti-cancer agents

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Abstract

Cancer recurrence following response to therapy suggests that resistant cells lay dormant and subsequently reactivate. The cancer stem cell paradigm explains why tumors typically consist of a large therapy sensitive compartment, and a smaller compartment with profound intrinsic resistance. Here we examine co-expression of tissue stem cell markers (CD90, CD117, CD133) and cytokeratin in previously untreated non-small cell lung cancer (NSCLC). In normal lung (NL), we assign a provisional ~~profile~~ to resting stem cells (low scatter, cytokeratin-and either $CD90^{dim}$/CD133+, or CD117+). Progenitors share this phenotype but are morphologically complex, downregulating CD90 as they gain cytokeratin. This pattern is retained in well-differentiated NSCLC, but is deranged in poorly-differentiated NSCLC, the most common pattern being overexpression of cytokeratin on stem/progenitors. Stem cells and progenitors are present at ~1% and 10% in NL and NSCLC, respectively. Constitutive MDR was present in ~6% of well-differentiated and ~50% of poorly differentiated tumors. We hypothesize that among the minority of tumor cells capable of propagating a tumor, only those that self-protect survive therapy. Of ~~these~~, only ~~cells~~ which, like normal stem cells, are predominantly resting, cause recurrence after remission. The therapeutic index of antineoplastics becomes one of sensitivity of cancer and normal stem cells, which are protected by the same mechanisms.

1. Introduction

ABC transporters are highly conserved and represent a major protective mechanism for barrier tissues as well as adult tissue stem cells [1–9]. Survival of tissue stem cells is essential to tissue maintenance and repair, and constitutive MDR activity is thought to be one of several protective mechanisms by which normal tissue stem cells guard themselves form from toxic insults, including those resulting from damage by chemotherapeutic agents [10]. Moreover, in the absence of tissue stem cell specific markers, the activity of these transporters has been exploited to obtain enriched populations of tissue stem cells [5,11,12]; the efflux or exclusion of fluorescent MDR substrates such as rhodamine 123 (ABCB1 substrate, R123dull/dim phenotype) and Hoechst33342 (ABCG2 and to a lesser degree ABCB1 substrate, Side Population (SP) phenotype) are frequently used in fluorescence activated cell sorting of hematopoietic and non-hematopoietic tissue stem cells.

The unique insight which we derive from the study of adult tissue stem cells and bone marrow derived stem cells [5,11] is that drug resistance, mediated in large part by ABC transporters, is a normal physiologic self-protective mechanism, which we hypothesize is retained by the nascent neoplasm upon transformation of the tissue stem cell [10]. The notion that the cancer-initiating cell may have constitutive drug resistance predicts the persistence of a therapy resistant stem cell-like fraction following apparently successful cytotoxic cancer therapy ~~resulting in the marked shrinkage or even disappearance of measurable tumor~~. Therapies that target proliferating cells, enzymes, growth receptors, adherence molecules, or signaling molecules in metabolically active cells may be highly effective at debulking tumors and dispatching tumorigenic cells without stem cell like properties, but they will consistently fail to eradicate the rare tumor cell fraction that shares protective mechanisms with their normal counterparts.

The cancer stem cell hypothesis attempts to explain the origin of cancer and identifies the cancer initiating cell as a transformed

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tissue stem cell. However, human cancers are heterogeneous and it is not always possible to apply a rigorous classification into stem, progenitor and mature compartments on the basis of differentiation marker expression and morphology. In normal adult tissues, stem cells are small resting undifferentiated cells which are confined to anatomic niches in which they are protected from toxic insults by both interaction with niche cells and by intrinsic mechanisms such as MDR transporter activity and detoxifying enzymes. When they are driven into proliferation, they replicate asymmetrically, giving rise to a stem cell daughter which remains in the niche, and a progenitor daughter (or transit amplifying cell) which migrates out of the niche, proliferates and efficiently gives rise to mature post mitotic cells with tissue specific characteristics. In doing so, the progenitor and its progeny progressively lose the markers of "stemness" (including self renewal and self-protection) as they gain tissue specific markers. However, there are variations on this theme, in which cells with mature function, such as hepatocytes, given appropriate stimuli, can revert to progenitor status and mediate tissue regeneration. It is also worthwhile to distinguish between three partially overlapping roles of tissue stem cells. The first role, organogenesis, is largely complete at birth, with the exception of the breast and prostate, which develop at puberty under hormonal influences. The second role, tissue maintenance, is a continuous process that proceeds at a rapid pace in some organs (skin, gut, blood) and at a glacial pace in others (nerves). Finally, adult tissue stem cells mediate tissue repair after injury, a function that is better developed in some organs (liver, blood) than in others (nerve). Whatever the role, the adult tissue stem cell is characterized by its anatomic location, and ability to self-replicate, self-protect, and give rise to further differentiated progeny of high proliferative capacity.

Returning to the cancer stem cell hypothesis, a strong analogy can be made between tumor growth and normal tissue growth in that: 1) The majority of tumor cells are post mitotic (non tumorigenic); 2) In order to be tumorigenic, at least a fraction of cells must be capable of sustained self-renewal (i.e. not loose proliferative capacity as a function of proliferative history); 3) In order to survive the toxic insults of therapy, a proportion of cells must retain or develop self-protective mechanisms (drug transport and metabolism). Fig. 1 shows a schematic representation of the cancer stem cell hypothesis. A mutated stem cell is shown at the ~~top of the chain~~, characterized by its resting protected state. These cells and their progeny, active, tumorigenic progenitor cells have already departed from the normal schema, as they retain the capacity for self renewal, as well as the ability to dedifferentiate into resting a resting protected phenotype. These last two points come from our experimental data showing that purified large, stem/progenitor marker positive (CD90), proliferating, MDR negative tumor cells are tumorigenic at very high frequency, and give rise to tumors as heterogeneous as those from which they were purified, including a small proportion of stem/progenitor marker+, MDR+ resting cells [13]. In this report we will further explore the expression of stem and progenitor cell markers and MDR activity in lung cancer and normal lung tissue, with emphasis on reconciling the cancer stem cell paradigm with both low and high grade malignancies.

2. Methods

2.1. Samples and sample preparation

Single cell suspensions were obtained from normal lung (adjacent grossly normal tissue from lung cancer patients = 17) and malignant adult lung tissues (n = 20). Specimens were collected in accordance with a protocol approved by the University of Pittsburgh Internal Review Board. Tissues were minced with paired scalpels, digested with type I collagenase and disaggregated through 100 mesh stainless steel screens (4% in RPMI 1640 medium, Sigma Chemicals, St. Louis MO) [14]. Ten to 500 million viable cells were recovered from a 5–10 mm^3 specimens of tumor or normal lung parenchyma. Viable cells of single cell suspensions obtained from adjacent normal parenchyma, tumor and pleural effusions were concentrated and separated on a ficoll/hypaque gradient.

2.2. Staining and flow cytometry

Single cell suspensions were stained according to a protocol described in detail elsewhere [15]. Five minutes prior to staining with fluorochrome-conjugated monoclonal antibodies, neat mouse serum (5 uL) was added to each cell pellet to minimize non-specific antibody binding. Prior to cytokeratin staining, cells were stained for surface markers (2 uL each added to the cell pellet, 15–30 min on ice), and fixed with ~~2~~1% methanol-free formaldehyde. Cells were then permeabilized with 0.1% saponin (Beckman Coulter, Fullerton, CA) in phosphate buffered saline with 0.5% human serum albumin. Antibodies and dyes used in these studies included: Pan cytokeratin-FITC (Beckman Coulter, Cat. No. IM2356), CD90-biotin (BD, Cat. No. 555594), Streptavidin-ECD (Beckman Coulter, Cat. No. IM3326), ABC G2-PC5 (Chemicon, Temecula CA, Cat. No. MAB4155PC), CD117-PC7 (Beckman Coulter, Cat. No. IM3698), CD133-APC (Miltenyi Biotech, Cat. No. 120001241), HEA-APC (Miltenyi

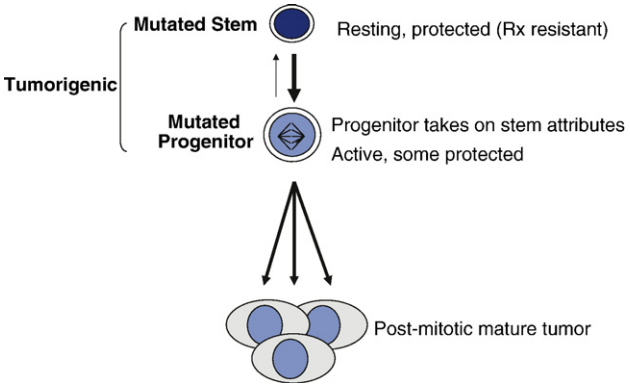

Fig. 1. The cancer stem cell hypothesis attempts to explain the origin of cancer and identifies the cancer initiating cell as a transformed tissue stem cell. ~~However,~~ human cancers are heterogeneous and it is not always possible to classify them into stem, progenitor and mature compartments on the basis of differentiation marker expression and morphology. That both stem and progenitor fractions are shown as tumorigenic is derived from our findings in breast cancer [13], as is the bidirectional path between stem and progenitor cells.

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Biotech, Bergisch Gladbach, Germany, Cat. No. 12000420), CD45-APCC7 (BD, Cat. No. 557833), DAPI (Sigma Chemicals, St. Louis MO, Cat.), Propidium iodide (Calbiochem, La Jolla, CA, Cat. No. 537059), Rhodamine 123 (Sigma Chemicals, St. Louis MO, Cat. No. R8004), Hoechst 33342 (Invitrogen, Carlsbad, CA, Cat. No. H3570). Fumitremorgin was purchased from Alexis, (Cat. No. ALX-350-127). Eight-color analysis was performed using the 3-laser, 9-color CyAn LX cytometer (Dako, Fort Collins, CO). Analysis requiring an ultraviolet laser was performed on a 3-laser 8-color Dako MoFlo. An effort was made to acquire a total of 5–10 million cells per sample at rates not exceeding 10,000 events/s. The cytometers were calibrated prior to each use using SpectrAlign beads (Dako, Cat. No. KO111) and 8-peak Rainbow Calibration Particles (Spherotech, Libertyville, IL, Cat. No. RCP-30-5A). Color compensation matrices were calculated for each staining combination within each experiment using single-stained mouse IgG capture beads (Becton Dickinson, Cat. No. 552843) for each antibody, and single-stained cells for rhodamine 123. Offline analysis was performed using a prototype version of Venturi, an analytical package utilizing parallel processing and designed specifically for multiparameter rare event problems (Applied Cytometry Systems, Dinnington, Sheffield, UK). In all analyses, doublets and clusters were eliminated using forward scatter peak width versus height as a discriminator. Propidium iodide staining was used to eliminate nonviable cells, DAPI staining was used to eliminate hypodiploid cells. The channel normally used for PE (FL2, 575 nm) was used to identify and eliminate autofluorescent cells.

2.3. Statistics

Statistical analysis was performed using Systat version 11 (Systat Inc, San Jose, CA). Stem and progenitor cell frequencies were log normally distributed and were log transformed prior to analysis by ANOVA.

3. Results

3.1. Identification of candidate stem progenitor and mature populations in lung tumors and normal lung tissue

Cancer stem cells were initially defined by a combination of function and expression of a particular profile of adhesion molecules. In breast and pancreatic cancer, ESA+ CD44+ CD24- tumor cells were 100 to 200-fold enriched in cells that were tumorigenic in an explant model [16,17]. Other investigators showed in human brain tumor that cells expressing CD133+, a marker originally described on hematopoietic progenitor cells, were enriched in tumorigenic cells [18]. We have added CD90 to the list of markers on tumorigenic breast cancer cells [13]. Strictly speaking, adult stem cells are normally resting cells which give rise to highly proliferative progenitor cells. In an attempt to shed light on the stem/progenitor distinction in cancer cells, we used 8-color flow cytometry to identify resting cells of low morphologic complexity (low light scatter), expressing the stem cell markers CD90, CD117 or CD133 in primary and metastatic lung cancer and in normal lung tissue. These are considered candidate cancer stem cells. Larger morphologically more complex cells expressing any of these same markers were considered candidates for cancer progenitor cells. Expression of intracellular cytokeratin was used to assess epithelial differentiation. All cells were gated to eliminate cell clusters, CD45+ hematopoietic cells, cells that had autofluorescence that spanned the wavelengths measured in FL1, FL2 and FL3 (525–620 nm), and DAPI^{dim} cells with <2 N DNA. Lymphocytes in the CD45+ fraction were used to define the light scatter properties of small resting cells. Three lung cancer pleural effusions, and 17 lung tumors with matched adjacent normal tissue were studied.

Fig. 2 shows representative profiles from normal lung tissue adjacent to a resected tumor, a histologically well differentiated

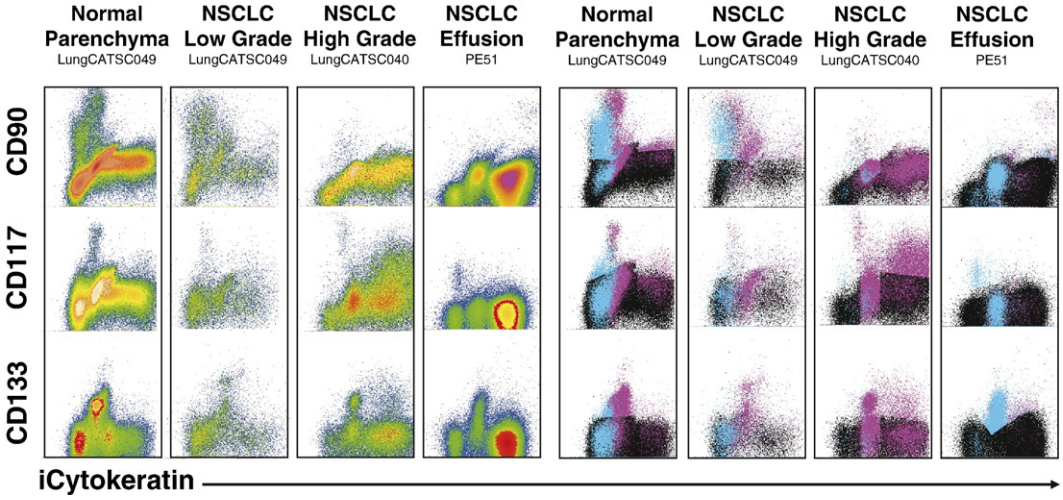

Fig. 2. Differentiation patterns in normal lung, and lung cancer tumor and pleural effusions. The stem/progenitor markers are plotted versus the epithelial differentiation marker cytokeratin. Left panels are density plots; right panels are color evented dot plots of the same data, where cyan dots represent cells of low light scatter (low morphologic complexity), and magenta dots represent cells of high light scatter (high morphologic complexity). Black dots represent cells that are CD90-, CD117-and CD133-. The dots were layered in the order of prevalence, with cyan on top, magenta in the middle, and black on the bottom.

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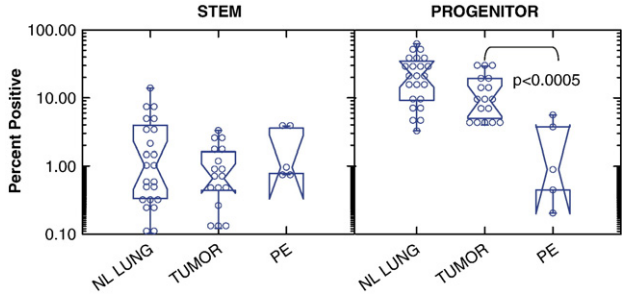

Fig. 3. Frequency of stem and progenitor cells in normal lung, non-small cell lung tumors, and malignant pulmonary effusions. Stem cells were defined as cells of low light scatter positive for one or more stem/progenitor cell markers (CD90, CD117, CD133). Progenitor cells were defined as stem/progenitor marker+ cells with high light scatter. Notched box plots display nonparametric (distribution-free) descriptive statistics. Individual data points are marked by open circles. The waist indicates the group median, the notch about the waist indicates the 95% confidence interval about the median. The hinges (upper and lower boundaries of the box) indicate interquartile distances. The whiskers (bars) give the ranges, exclusive of outliers.

non-small cell adenocarcinoma of the lung, a poorly differentiated non-small cell adenocarcinoma of the lung, and a pleural effusion of metastatic non-small cell lung cancer. All samples were freshly isolated from newly diagnosed previously untreated patients. The left panels show density plots of the stem cell markers versus the epithelial differentiation marker intracellular cytokeratin; in the right panels, the same parameters are displayed as color event profiles, where the cyan dots are ~~cells~~ small resting cells expressing any combination of CD90, CD117, and CD133; magenta dots are large morphologically complex cells expressing one or more stem cell marker; ~~and~~ black dots are cells without any stem cell marker expression. Disaggregated normal lung is a complex tissue comprised of small airways, alveoli and small vessels; this complexity is reflected in the stem-progenitor profile. The small resting cells are virtually all cytokeratin negative, with a major population of $CD90^{dim}$ cells and minor populations of CD117+ and CD133+ cells. CD133+ and $CD90^{dim}$ are sometimes coexpressed (not shown), but CD117 is a unique population. The progenitor fraction is more heterogeneous, with CD90+ and CD133+ cells decreasing in fluorescence intensity as they mature from cytokeratin dim to bright. Even more so than the resting cells, $CD90^{dim}$ and CD133 expression is well correlated, whereas CD117 is present on a unique population. Despite the obvious gross differences between tumor and adjacent lung tissue, the well differentiated (low grade) non small cell lung cancer shows a stem-progenitor profile virtually identical to the normal lung tissue, which was isolated from the same patient. The relationship between CD90 and cytokeratin expression is particularly interesting, showing four discrete populations evocative of a differentiation pattern. Small resting cells are virtually all $CD90^{dim}$ and cytokeratin negative. They appear to progress to larger $CD90^{bright}$ cytokeratin negative cells. As cytokeratin intensity increases from negative to dim to bright, the intensity of CD90 staining decreases. The vast majority of cytokeratin bright cells express no stem/progenitor markers. This apparent differentiation pattern is entirely absent in the examples of high grade non small cell lung cancer and malignant pleural effusions. Here small resting stem cell marker+ cells can express dim or bright cytokeratin and ~~the~~ a significant proportion of large stem cell marker+ cells (progenitor cells) are cytokeratin bright.

Fig. 3 shows summary statistics of the frequency of stem ~~cells~~ and progenitor cells in normal lung, lung tumors, and pleural effusions. Frequencies are expressed as a percent of singlet cells with DNA content ≥ 2 N. Stem cells were defined as cells with low light scatter (comparable to resting lymphocytes), expressing one or more stem/progenitor markers (CD90, CD119, CD133). Progenitor cells were stem/progenitor marker positive cells with high light scatter. Stem cell frequency was constant across normal lung and lung tumors (mean (lower, upper 95% confidence interval) = 0.92% (0.64, 1.34)). Progenitors comprised 18.9% (13.2, 27.1) of normal lung, 10.0% (6.97, 14.5) of tumor and 1.1% (0.20, 6.34) of pleural effusions. The progenitor content of pleural effusions was significantly lower than that of normal lung or lung tumor ($p < 0.0005$).

3.2. MDR activity and in low and high grade malignancies

MDR activity has been used to identify and isolate stem cell enriched fractions from a variety of tissues. Fig. 4 shows simultaneous measurement of the exclusion of two MDR substrates, Hoechst 33342 and rhodamine 123 on freshly isolated, therapy naïve non small cell lung cancer cells. Antibody stained ~~suspended~~ tumor cells were incubated simultaneously with Hoechst 33342 ~~(8 μM)~~ plus R123 ~~(0.13 μM) for 90 min at 37 °C~~. Propidium iodide ~~(PI, 10 μg/mL)~~ was added immediately before sample acquisition. All events were gated on ~~PI excluding (~~live~~)~~, non-hematopoietic ~~CD45-)~~ singlets ~~(doublet discrimination based on forward light scatter pulse analysis)~~. A total of 2.3 million cells were acquired.

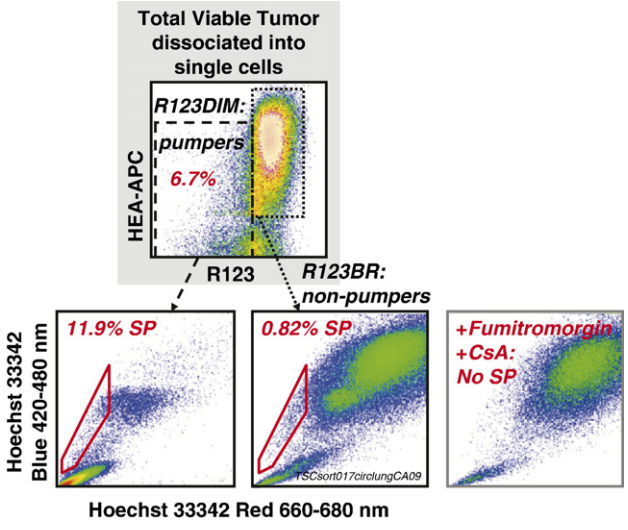

Fig. 4. ABCG2 and ABCB1 activity in freshly isolated therapy naïve non-small cell lung cancer. Antibody stained suspended tumor cells were incubated simultaneously with Hoechst 33342 (8 μM) plus R123 (0.13 μM) for 90 min at 37 °C. Propidium iodide (PI, 10 μg/mL) was added immediately before sample acquisition. All events were gated on PI excluding (live), non-hematopoietic CD45-) singlets (doublet discrimination based on forward light scatter pulse analysis). A total of 2.3 million cells were acquired.

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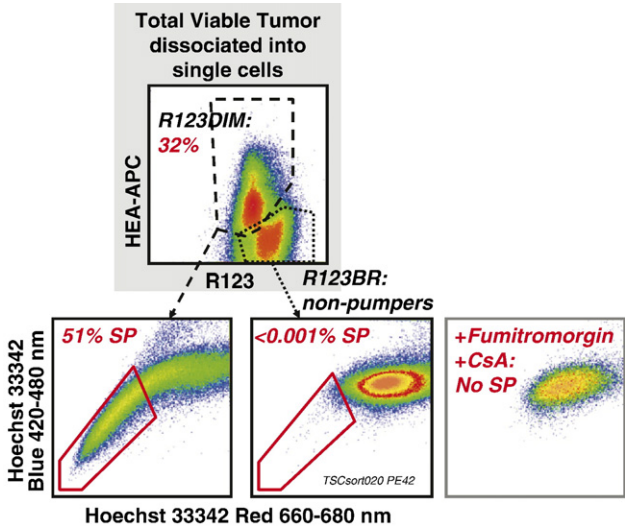

Fig. 5. ABCG2 and ABCB1 activity in freshly isolated therapy naïve malignant pleural effusion. Antibody stained density gradient separated pleural effusion cells were incubated simultaneously with Hoechst 33342 and Rhodamine123 as described in Fig. 4. Propidium iodide was used as a viability discrimination dye where all events were gated on PI excluding (live), non-hematopoietic CD45- singlets. A total of 3.9 and 2.6 million cells were acquired in 2 consecutive runs.

This untreated newly diagnosed moderately well differentiated adenocarcinoma of the lung has a small proportion (6.7%) of cells which constitutively efflux R123. R123 is mainly transported by ABCB1. R123 dim (pumping) cells were heterogeneous for the expression of the epithelial differentiation marker HEA. They contain a significant subpopulation (11.9%), which co-transports the ABCG2 substrate Hoechst 33342. Cells which constitutively transport Hoechst 33342 have been termed the Side Population (SP). The great majority of R123bright cells was HEA+ and was almost devoid of SP cells. The R123dim and SP phenotypes were completely abrogated by the addition of the MDR inhibitors fumitremorgin plus cyclosporine, supporting the MDR transporter specificity of this functional assay.

Fig. 5 shows an identical experiment performed using therapy naïve cells freshly isolated from a poorly differentiated, highly aggressive renal carcinoma metastatic to the lung. Unlike the previous example, ~~The~~ R123 efflux activity was present in a major population and exclusively confined to HEA+ cells. Among R123 effluxing cells, 51% co-transported Hoechst 33342. These data indicate a great expansion in the population with constitutive transporter activity, and breakdown in the orderly expression of stem cell-like transporter activity in differentiation marker negative cells.

4. Discussion

4.1. Tumor differentiation grade and the cancer stem cell paradigm

Current descriptions of the cancer stem cell hypothesis make good intuitive sense with low grade, well differentiated tumors, where the majority of cells are non-tumorigenic deranged cells with a gene expression profile ~~architectural~~ remnants reflecting their ~~tissue~~ of origin. Within such tumors, careful microscopic inspection will reveal a rare proliferating progenitor cell population, discernable as mitotic figures. Flow cytometric analysis of dissociated tumor examining millions of cells reveals a still rarer population of stem cell marker positive resting cells, a proportion of which also have constitutive MDR activity and detoxifying enzyme activity (e.g. aldehyde dehydrogenase). This is shown in schematic in the left panel of Fig. 6.

At first blush, high grade, poorly differentiated tumors do not seem to fit the cancer stem cell paradigm. Proliferating cells are common, the tissue of origin is often difficult, and sometimes impossible to discern as histological cues and differentiation markers are often lacking. Here it is useful to remember that cancer is a disease of mutation and selection, and there is no predicting how much or how little of the normal differentiation ~~pathway~~ will be retained. For lack of better terminology, poorly differentiated tumors have a large population of differentiation deranged cells with properties of both stem and progenitor cells; these cells express both the proliferative capacity of the progenitor cell and the self-renewal of the stem cell (Fig. 6 right panel). Whether they also retain the self-protection of the stem cell will determine whether they are intrinsically therapy resistant, or whether like some poorly differentiated hematologic malignancies, are exquisitely sensitive. It should be noted that neither of these scenarios in poorly differentiated disease preclude the retention of a vestigial population of tumor stem cells, with characteristic resting state ~~and~~ self-renewal and self-protective capacities. That these resting cells are present in poorly differentiated cancer is borne out by our experimental data (Fig. 2) as well as clinical findings. In lung cancer, patients with poorly differentiated tumors tend to have better initial response to chemotherapy than those with well differentiated disease, but paradoxically, have shorter survival [19].

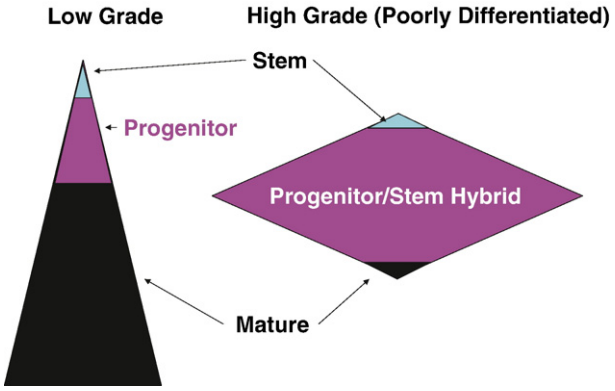

Fig. 6. Differentiation patters in well and poorly ~~differentiation~~ malignancies. Well differentiated malignancies resemble normal tissue and have distinct stem and progenitor compartments. Poorly differentiated malignancies retain a rare resting stem population, but lack a large population of cells bearing recognizable signs of maturation. Poorly differentiated cancers with a progenitor fraction that has acquired stem cell-like protective mechanisms (high MDR) will present as therapy resistant. Poorly differentiated cancers without a significant protected progenitor fraction proliferate aggressively but respond well to treatment. In both cases, resting cancer stem cells are spared by therapy.

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The most important contribution of the cancer stem cell hypothesis is the recognition that characteristics retained from normal tissue stem cells, or acquired as the result of multiple mutations, guarantee that a proportion of tumor cells will be intrinsically at least as therapy resistant as ~~their~~ normal tissue and hematopoietic ~~counterparts~~. The selective pressure of therapeutic agents may further hone this resistance, but an intrinsically resistant population has been present in the tumor from its inception. Specifically, the characteristics that mediate intrinsic therapy resistance are: 1) The resting state, which renders cells resistant to agents dependent on DNA synthesis or high metabolic activity; 2) The lack of differentiation markers such as receptors (ER/PR, EGFR, VEGFR) and tissue specific proteins (tyrosine kinase) which constitute targets of therapy in more mature tumor cells; 3) Constitutive expression of MDR transporters; 4) Constitutive activity of detoxifying enzymes; 5) Resistance to apoptosis mediated by interaction of surface receptors and protective cells in the tumor environment (reviewed in [20]).

4.2. Tumorigenicity versus tumor survival after therapy

It has long been recognized that only a fraction of tumor cells are tumorigenic. Successful cancer therapy requires eliminating all tumor populations from three distinct compartments: 1) Mature post mitotic tumor cells, which often interfere mechanically and biochemically with normal function; 2) Rapidly proliferating progenitor cells that are the source of bulky tumor; and 3) The rare resting, protected compartment of the tumor identified as cancer stem cells. It is important to distinguish between tumorigenic cells, which are not necessarily stem cells, and stem cells, which are not necessarily protected. The real culprit, and the population that routinely evades therapy is the tumorigenic, protected cell, and this is often a resting cell.

In support of this notion, we have previously shown that not all tumorigenic cells are stem cells [13], as defined by both expression of accepted stem cell markers and resting morphology. Neither is tumorigenicity enriched in tumor cells with constitutive MDR activity [21] nor expression [13]. In contrast to tumor-derived cells, sorting for the constitutively MDR active "side population" greatly enriches normal stem cells from a variety of normal tissues (cited above [4–9]).

So why have experiments testing tumorigenicity of tumor derived side population cells failed to show enrichment of tumorigenic cells within this population [21]? Fig. 7 shows graphically that the extent to which MDR activity and clonogenicity overlap will determine the effect of side population enrichment on tumorigenicity. In normal tissue ~~stem cells~~ not all stem cells are MDR protected (and therefore SP+), and not all SP+cells are stem cells, but the overlap is considerable (left panel). This appears not to be the case in tumors, where both MDR activity and tumorigenicity appear to be rare events with limited overlap. Despite the uncoupling of MDR protection and tumorigenicity, there remains a rare MDR protected tumorigenic population which we propose to be the cell which evades apparently successful therapy.

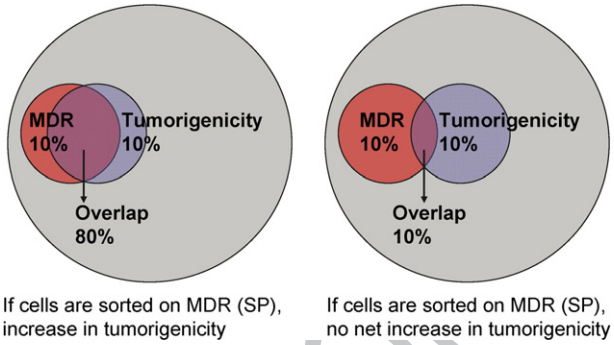

Fig. 7. Two scenarios concerning the relationship between tumorigenicity/clonogenicity and constitutive MDR expression. On the left the frequency of MDR+ and tumorigenic cells are both 10%, but they overlap substantially. As in normal tissues, sorting MDR+ cells would yield a substantial enrichment in clonogenic cells. On the right MDR+ cells and tumorigenic cells also constitute 10% of the tumor population, but they only overlap by 10%. Sorting MDR+ cells in this case would result in no enrichment of tumorigenic cells. Importantly, in both cases a fraction of tumorigenic cells are protected by constitutive MDR activity and therefore inherently therapy resistant.

4.3. Therapeutic index revisited

MDR activity has been proposed as a major mechanism responsible for sparing normal tissue stem cells from the effects of chemotherapy, which often kills both the bulk of tumor cells as well as actively metabolizing normal cells in the bone marrow, skin and gut. Therapeutic agents, in fact, are dosed to prevent irreversible damage to normal tissue. This translates to preventing irreversible damage to tissue stem cells. A dramatic example can be found in chemotherapy-induced alopecia, which results from damage to the progenitor cells of the hair follicle. These cells are sensitive to antimitotic and antimetabolic agents because of their rapid turnover. However, alopecia is reversed on cessation of therapy, precisely because the four distinct cell types which comprise the follicle, as well as skin epithelial cells themselves, are all derived from a common progenitor, which itself is derived from an MDR-protected epithelial stem cell.

Given the paradigm shown in the left hand panel of Fig. 6, the closer resting cancer stem cells resemble their normal counterparts, the less likely MDR substrate drugs, or non-substrate agents that target proliferating cells, receptors or enzymes in metabolically active cells, will be effective. Herein lies the conundrum of therapeutic index. The efficacy of an anti-neoplastic agent or regimen is judged by its ability to shrink tumors without irreversible damage to normal tissue. However, successful treatment is often defined as the ability to achieve a durable remission. The very fact that cancers can and do relapse after apparently successful therapy indicates the survival of an occult treatment-resistant tumorigenic population. Therapeutic index must therefore be redefined as the differential toxicity to cancer stem cells, relative to tissue stem cells. This is a much more difficult problem because it requires agents which can exploit biological differences between cancer and normal stem cells, rather than the numerous disparities between metabolically active tumor cells and resting normal stem cells.

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Multiple Drug Resistance in Cancer Revisited: The Cancer Stem Cell Hypothesis

Vera S. Donnenberg, PhD, and Albert D. Donnenberg, PhD

The failure to eradicate cancer may be as fundamental as a misidentification of the target. Current therapies succeed at eliminating bulky disease but often miss a tumor reservoir that is the source of disease recurrence and metastasis. Recent advances in the understanding of tissue development and repair cause us to revisit the process of drug resistance as it applies to oncogenesis and tumor heterogeneity. The cancer stem cell hypothesis states that the cancer-initiating cell is a transformed tissue stem cell, which retains the essential property of self-protection through the activity of multiple drug resistance (MDR) transporters. This resting constitutively drug-resistant cell remains at low frequency among a heterogeneous tumor mass. In the context of this hypothesis, the authors review the discovery of MDR transporters in cancer and normal stem cells and the failure of MDR reversal agents to increase the therapeutic index of substrate antineoplastic agents.

Keywords: *Cancer stem cell; multiple drug resistance; chemotherapy; oncogenesis.*

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The failure to eradicate most cancers may be as fundamental as a misidentification of the target. Our current therapies succeed at eliminating bulky disease and rapidly proliferating cells but often miss a tumor reservoir that is the source of disease recurrence and metastasis. Recent advances in the understanding of normal tissue development and repair provide a basis for revisiting the process of oncogenesis, tumor heterogeneity, and drug resistance.

From a pharmacological perspective, where emphasis is placed on developing therapies and understanding treatment failure, the tumor stem cell hypothesis provides several new insights that may help us rethink strategies for cancer treatment. Understanding the central role played by multiple drug resistance (MDR) transporters in the protection and self-renewal of normal and cancer stem cells may allow us to identify differences that can be exploited therapeutically. Recognizing that normal stem cells in individual tissues differ with respect to damage tolerance and degree of multipotentiality may translate into differential drug susceptibilities and metastatic potentials of cancer stem cells, depending on the tissue of origin.

This review will attempt to elaborate on the tumor stem cell hypothesis by focusing on the discovery of MDR transporters in neoplastic cells and on the growing body of evidence that these transporters are also an essential feature that enables tumor stem cells to circumvent therapy.

DISCOVERY OF MDR PUMPS AND IN VITRO STUDIES OF MDR

Resistance to chemotherapy was recognized as an impediment to efficacious cancer treatment in the earliest stages of anticancer drug development.[1] Surprisingly, cancer cell lines selected for resistance to specific compounds frequently demonstrated cross-resistance to a broad spectrum of structurally unrelated agents.[2] In a first attempt to account for the mechanism of cross-resistance, Ling and Thompson showed that resistant cells displayed reduced plasma membrane permeability to cytotoxic compounds.[3]

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The advent of molecular approaches led to the isolation of the first candidate genes for MDR. Roninson and colleagues hypothesized that resistance in drug-selected cancer cell lines arose from amplification of a gene product or gene products capable of altering the plasma membrane. Noting cytogenetic abnormalities common to resistant hamster cell lines, they cloned these amplified fragments and showed that the gene or genes encoded in these fragments were amplified in resistant but not susceptible cells. Removal of selective pressure led to reversion to the drug-sensitive phenotype and coincided with the loss of the amplified DNA.[4] Further experiments revealed 2 genes, now recognized as the hamster homologs of the human *MDR1* and *MDR2*.[5,6] In 1986, Gros and colleagues transfected the gene now known as *MDR1* into drug-sensitive hamster cells. Importantly, they showed that gene duplication or mutations were not required for the acquisition of the multidrug-resistant phenotype.[7] The *MDR1* gene product is now known as P-glycoprotein (ABCB1). Twentyman and colleagues demonstrated that addition of verapamil (now recognized to be a competitive inhibitor of several MDR transporters) significantly increased the susceptibility of drug-resistant human lung cancer cell lines,[8] at once providing a means of verifying MDR activity in vitro and a potential therapeutic avenue for increasing the sensitivity of cancer cells to MDR substrate drugs. ABCB1 did not account for all forms of multiple drug resistance, and additional transporters were identified, among them ABGG2, first described as mitoxantrone resistance[9] and later as breast cancer resistance protein (BCRP).[10] Today, ABCB1 and ABCG2 are recognized as belonging to a family of at least 48 human ABC transporters involved in a variety of essential cellular transport processes.

ROLE OF MDR TRANSPORTERS IN NORMAL TISSUE STEM CELLS

The fluorescent dyes Hoechst 33342 and rhodamine 123 (R123) are now known to be substrates for the MDR transporters ABCG2 and ABCB1, respectively. This explains several important earlier findings: 1) Hoechst 33342 dim murine bone marrow cells are greatly enriched for high proliferative potential colony-forming cells;[11] R123 dim bone marrow cells protected mice after lethal irradiation;[12] and provided a 240-fold enrichment in long-term hematopoietic colony-initiating cells as compared to unfractionated bone marrow.[13] However, the critical proof of constitutive upregulation of MDR transporters in primitive hematopoietic stem cells came from Goodell et al, who showed that 10% of Sca-1+, lineage-negative murine bone marrow cells (a phenotype used to define early hematopoietic stem cells) were also Hoechst 33342 dim.[14] They termed this small cell subset the *side population* (SP), after their distinctive flow cytometric profile. Compared to whole bone marrow, SP cells, which composed only 0.1% of bone marrow cells, were 1000-fold enriched with respect to cells able to repopulate lethally irradiated mice. Goodell et al showed that the SP phenotype was abrogated by culturing cells with verapamil, an MDR inhibitor, thus demonstrating that a constitutively active MDR transporter was responsible for exclusion of the Hoechst dye. Sorrentino and colleagues elegantly worked out the details, showing that transfection of ABCB1 (P-glycoprotein) into normal murine marrow increased the SP phenotype by 2 orders of magnitude,[15] ABCG2 knockout abrogated the side population,[16] and both ABCB1 and ABCG2 are constitutively active in SP cells.[17]

Although hematopoiesis remains the leading paradigm for tissue differentiation and replacement, the study of adult tissue stem cells has gained momentum with the emergence of the field of regenerative medicine. The potentiality and plasticity of tissue stem cells that mediate tissue repair and maintenance constitute an area of intense study. MDR transporter activity, in the form of the SP, has provided the principal means to recognize and purify such tissue stem cells.[18] Although little is known about the role of MDR transporters in adult tissue stem cells, we hypothesize that they follow the hematopoietic paradigm, affording resting stem cells a means of protection that allows them to survive toxic insults that destroy cycling progenitor cells and mature tissue.

ATTEMPTS TO USE MDR INHIBITORS THERAPEUTICALLY

The discovery of the molecular mechanism of cross-resistance led immediately to attempts to block MDR transporters with putative reversal agents. Reversal of MDR in vitro was easily attained with a variety of inhibitors. However, in vivo, MDR reversal in the clinical setting has proven to be much more difficult. Thus, intracellular concentrations of adriamycin, vincristine, and etoposide were all significantly increased in 7 human non-small-cell lung cancer cell lines cocultured in the presence of verapamil (2.2-6.6 μm).[19] Similarly, verapamil increased drug sensitivity of ovarian cancer cells lines rendered resistant by culture in the presence of doxorubicin. However, an early indication that in vitro drug selection provides a less than ideal model for in vivo cross-resistance was the observation that primary tumor cell lines isolated from pa-

tients with doxorubicin-refractory disease failed to demonstrate drug resistance in vitro.[20] This observation will be discussed further in the context of innate versus acquired resistance. Furthermore, in ovarian cancer patients receiving verapamil to plasma levels sufficient to cause inhibition of MDR-mediated adriamycin resistance in vitro (720-2767 ng/mL, = 1.5-5.6 μm), there was no evidence of enhanced response or toxicity of adriamycin coadministered at 50 mg/m^2.[20] Similarly, in a murine model of adriamycin resistance, continuous infusion of verapamil at the maximally tolerated dose failed to increase the sensitivity of resistant P388 lymphoma cells to adriamycin, despite a strong in vitro effect.[21]

Although there is still no clear-cut explanation for the failure of verapamil to act as a reversal agent in vivo, substantially different results were obtained with cyclosporine, an agent with a 10-fold higher affinity for the MDR on- and off-sites than the chemotherapeutic agent vinblastine.[22] When cyclosporine was given to patients with a variety of refractory cancers in combination with etoposide, cyclosporine levels ranging from 297 to 5073 ng/mL (0.25-4.2 μm) were obtained. Cyclosporine administration had a marked effect on the pharmacokinetics of etoposide, with a doubling of the area under the plasma concentration-time curve. As a result of both decreased renal and nonrenal clearance, a 50% dose reduction was required in patients with normal renal and hepatic function. Toxicities were tolerable but consistent with down-modulation of MDR function in the blood-brain barrier, bone marrow, and biliary tract. Unfortunately, the most critical parameter, intratumor etoposide levels, could not be determined by these studies. Clinical results were modest in this phase I trial of refractory patients, with demonstrable tumor regression in 4 of the 25 patients who attained cyclosporine plasma concentrations in excess of 2000 ng/mL.[23]

Convincing evidence that administration of an MDR reversal agent could increase the intratumor concentration of a chemotherapeutic agent was provided by Bates and colleagues,[24] who used the imaging agent Tc-99m sestamibi, an MDR1 substrate, to measure MDR activity in vivo. Using this technique, they demonstrated the efficacy of the nonimmunosuppressive cyclosporine analog PSC 833 (Valspodar) to reverse MDR activity in vivo. Ten patients with metastatic renal or adrenocortical carcinoma were imaged prior to therapy, 1 day after completing a course of vinblastine and on coadministration of vinblastine and PSC 833. Time activity curves and areas under the curve were obtained for tumor, liver, lung, and myocardium. Myocardium was used as a reference tissue to measure sestamibi uptake in the absence of MDR activity. During the coadministration of PSC 833, tumor visualization was markedly enhanced due to inhibition of MDR-mediated sestamibi efflux, suggesting that intratumor vinblastine concentrations were likewise increased.

Targeting MDR substrate drug directly to the tumor has been modeled using immunoliposomes loaded with doxorubicin and KG-1a leukemia cells. The liposomes were targeted to CD34 expressed on the leukemia cells using an anti-CD34 monoclonal antibody. Immunoliposomal doxorubicin showed a higher cytotoxicity against KG-1a cells than did nontargeted liposomal doxorubicin but failed to overcome doxorubicin resistance. Analysis of liposome-target cell interactions revealed that bound liposomes were not internalized. Thus, the increased cytotoxic effect may have been due to drug release proximal to the cells but not to a breach of membrane-associated MDR transporters.[25]

Further trials of first-generation modulators such as verapamil, quinidine, and cyclosporine proved them to be either inefficacious or associated with unacceptable toxicities. The limited clinical utility of the second- and third-generation MDR inhibitors such as PSC 833, GF120918, VX-710 (Biricodar), and LY335979 for potentiating antineoplastic agents may also be explained in part by multiple and redundant cellular mechanisms of resistance, unfavorable alterations in the pharmacokinetics of cytotoxic agents, and attendant toxicities associated with the systemic inhibition of MDR function. Since MDR transporters are themselves redundant with overlapping activities, specific inhibition of 1 transporter may leave drug resistance essentially intact.[17] The take-home message of these studies is that MDR reversal agents can be used to increase the plasma concentration of a variety of antineoplastic agents but not to increase their therapeutic index (reviewed by Tan et al[26]). The hypothesis that cancer arises uniquely from the mutation of tissue stem cells provides a theoretical framework for understanding this important observation.

CANCER STEM CELL HYPOTHESIS

The cancer stem cell was first proposed by Fiala in 1968.[27] Although modern concepts of stem cell biology were absent, the cancer-initiating cell was clearly hypothesized to be a "stem cell unable to differentiate." The cancer colony assay proposed by Hamburger and Salmon in the late 1970s introduced the concept that only a small proportion of cancer cells, cancer stem cells, are tumorigenic, and the authors identified these cells as the essential target of therapy.[28] However, re-

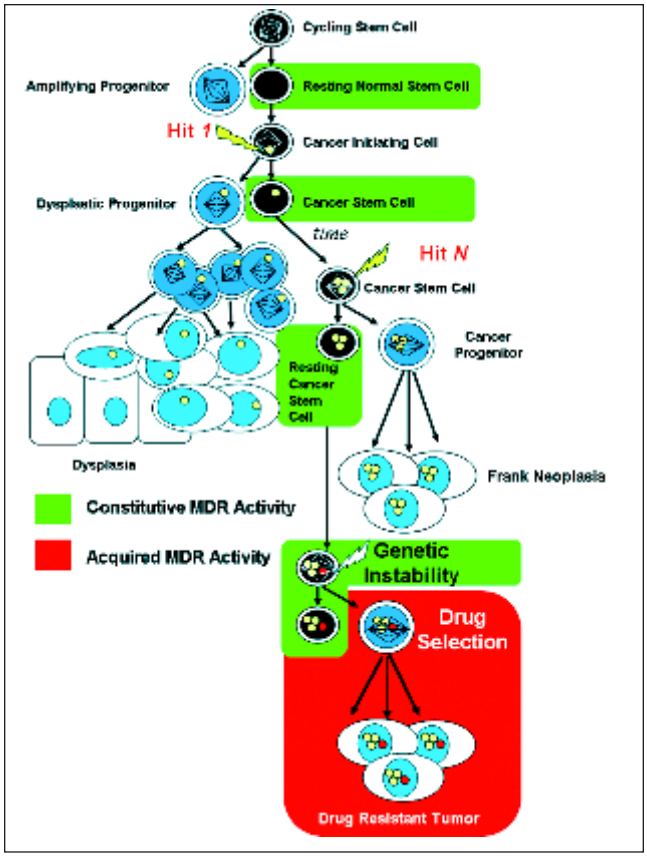

Figure 1. Constitutive and acquired multiple drug resistance (MDR) and the cancer stem cell hypothesis. The cancer stem cell hypothesis posits that the cancer-initiating cell is a tissue stem cell, where a stem cell is understood to be a cell that can self-renew, self-protect, and give rise to progenitors of high proliferative activity (amplifying or progenitor cells), which in turn produce mature progeny. The key features of a stem cell, as distinguished from a progenitor cell, are self-renewal, self-protection, damage tolerance, and a developmental state earlier than the highly proliferative progenitor cell that gives rise to fully differentiated daughters. The multistep process of neoplastic transformation begins with a mutation (yellow circle, hit 1) to a cycling tissue stem cell. Normally, tissue stem cells have constitutive MDR activity, but upon entry into the cell cycle, MDR is transiently downregulated, resulting in a window of vulnerability to DNA damage. In a process known as asymmetrical division, cycling stem cells self-replicate, giving rise to an MDR-protected resting stem cell and a drug-sensitive amplifying progenitor cell of high proliferative capacity. The damaged daughter stem cell reverts to an MDR-protected state when it exits the cell cycle, while the damaged progenitor proliferates and gives rise to drug-sensitive dysplastic cells. Additional mutations (hit N) and genetic instability accumulated by the damaged stem cell result in further growth dysregulation and the emergence of frank neoplasia. Throughout this process, the cancer stem cell remains protected and rarely enters the cell cycle. The bulk of the tumor mass is generated by mitotically active, drug-sensitive amplifying progenitor cells. Exposure to MDR substrate antineoplastic agents results in the elimination of drug-sensitive tumor but not resting tumor stem cells. It also imposes selective pressure for mutations that may result in overexpression of MDR transporters (red circle) and the acquired phenotype of drug resistance in tumor progenitor cells and their progeny. The most important concepts to emerge from this model are (1) MDR is constitutively expressed at high levels in tissue stem cells and therefore in the nascent cancer cell, (2) cancer results from accumulated mutations at the stem cell level, and (3) the cancer stem cell hypothesis posits that the clinically relevant target of therapy is a resting cell with drug resistance that is not dependent on therapy-induced gene duplication or translocation.

cent advances in regenerative biology have allowed tumor growth to be understood in the context of the dysregulation of normal tissue replacement and wound healing.

The modern revival of the tumor stem cell paradigm originated in the laboratory of Dr Irving Weissman,[29] who first isolated the multipotential hematopoietic progenitor cell.[30] Knowledge of the central role that MDR transporters play in protecting normal stem cells has allowed us to further refine this hypothesis and add new insights that may prove relevant to explaining treatment failure, late recurrence, metastasis, and tissue-specific differences in cancer incidence. Such knowledge may also guide us to design rational therapies that take into account similarities and differences between cancer and normal stem cells.

Given the central role of MDR transporters in protecting normal and neoplastic cells, the cancer stem cell hypothesis provides a unified explanation for the successes and failures of cytotoxic antineoplastic therapy (detailed in Figure 1). Namely, the most important target, the resting cancer stem cell, is spared along with its normal tissue stem cell counterparts. On a populational level, different malignancies may appear to be heterogeneous with respect to drug responsiveness. Cancers that respond to therapy initially may appear to acquire drug resistance during the course of treatment. Other cancers may appear to be intrinsically resistant. The cancer stem cell hypothesis posits that in both instances, the resting cancer stem cell, which is both the cancer-initiating cell and its source of replenishment under selective pressure, has innate drug resistance by virtue of its resting stem cell phenotype. Acquired drug resistance in more differentiated cancer cells, through gene amplification or rearrangement, may contribute to an aggressive phenotype, but it is not the primary reason for cancer recurrence or spread after therapy.

As detailed above, one of the defining characteristics of adult tissue stem cells is their constitutive resistance to environmental toxins, including most chemotherapeutic agents. In fact, dose-limiting toxicities of many antineoplastic agents occur precisely at drug concentrations that damage normal tissue stem cells. The constitutive drug resistance of normal tissue stem cells is mediated by MDR transporters and detoxifying enzymes. DNA repair mechanisms, tolerance to damage (ie, resistance to apoptosis), and telomerase activity

also contribute to the stability of normal tissue stem cells.

INNATE VERSUS ACQUIRED MDR IN TUMOR CELLS

In light of the cancer stem cell hypothesis, it is worthwhile to reexamine issues of drug resistance, cross-resistance, and the failure of MDR reversal strategies. The compounds that have been the most studied clinically as reversal agents are verapamil and cyclosporine and its analogs. Despite some promising results in hematological malignancies, the outcomes achieved when MDR blockers were coadministered with substrate drugs have been disappointing. This failure may be partially explained by the redundancy of the individual transporters within the MDR phenotype together with several other resistance-related proteins expressed in solid tumors (eg, glutathione S-transferase, metallothionin, O6-alkylguanine-DNA-alkyltransferase, thymidylate synthase, dihydrofolate reductase, heat shock proteins) or other factors contributing indirectly to resistance such as vascularization.[31] However, it follows from the cancer stem cell hypothesis that systemic administration of an efficacious reversal agent would render tumor and normal tissue stem cells equally susceptible to the chemotherapeutic agents, offering no net gain in therapeutic index.

As discussed above, the discovery of the first MDR transporter began with the observation of gene amplification in hamster cells selected in vitro for drug resistance.[4] Removal of the drug used for selection resulted in the outgrowth of cells without amplified MDR genes and loss of the multiple-resistant phenotype. However, in vivo drug resistance is not dependent on prior drug exposure, as was demonstrated using the tumor cell culture assay to culture lung cancer cells.[32] Current knowledge of regulation of MDR activity in stem cells and their progeny allows reconciliation of these findings. Drug resistance is an innate characteristic of the resting tumor stem cell but must be acquired by more differentiated tumor cells through gene amplification or rearrangement. The idea that transforming events in cancer lead to the juxtaposition of MDR and active genes through gene rearrangement is consistent with Roninson's findings in cell lines but is essentially an epiphenomenon according to the stem cell hypothesis. The cancer stem cell expresses constitutive MDR activity, which is independent of drug exposure, and is downregulated in more differentiated tumor progeny. It has been proposed that selective pressure imposed by chemotherapy leads to both mutation and secondary genetic changes, including MDR upregulation in the bulky tumor.[33] However, unless these changes occur in the self-renewing tumor stem cell compartment, the limited proliferative capacity of the bulky tumor ensures that they are self-limiting. Thus, the major barrier to therapy is the quiescent tumor stem cell with constitutive MDR.

IMPLICATIONS FOR THERAPY

Hematologic malignancies stand out among the cancers that are sometimes susceptible to cure. Allogeneic hematopoietic stem cell transplantation has been particularly successful[34,35] because stem cell rescue with donor hematopoietic stem cells obviates the need to spare normal hematopoiesis. Although skin, gut, and other tissues with rapid turnover are acutely affected by high-dose therapy, their constitutively drug-resistant stem cells quickly replenish the damaged tissue. Even if the transformed leukemia stem cells have evolved mechanisms that render them more protected from toxic insults than normal hematopoietic stem cells, immune recognition of minor histocompatibility antigens expressed on leukemia stem cells, but not on repopulating donor hematopoietic stem cells, provides the coup de grace in a process now recognized as the graft versus leukemia effect.[36]

Unfortunately, no analogous ability now exists to rescue nonhematopoietic stem cells following stem cell ablative therapy. If the proposed relationships between normal and neoplastic stem cells prove correct, the inescapable conclusion is that systemic cytotoxic therapies are doomed to failure because regimens that spare resting normal stem cells will also likely spare resting tumor stem cells. Successful therapy awaits the discernment of biological and immunological differences between the tumor and normal stem cells and the exploitation of the hypothesized window of vulnerability (Figure 1) that exists when the cancer stem cell is transiently recruited into the cell cycle.

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